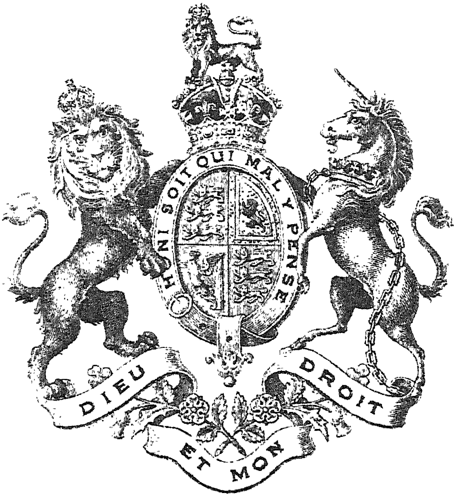

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In consequence of these facts there is at present no satisfactory evidence of the nature of the compounds containing a large proportion of the nitrogen in the green leaves of any plant. The highly specialized physiological functions of the leaf justify the expectation that it may contain constituents chemically unlike those found in other parts of the plant or in the cells of animal tissues. In how far such constituents belong to types of compounds peculiar to the leaf, or to types already well known, is scarcely known at present. It suffices merely to mention chlorophyll to call to mind one such substance. No one can foretell how many others which are likewise peculiar to the leaf may be present.

It is highly probable that the leaf is the seat of protein synthesis as already it is known to be the seat of carbohydrate synthesis. The close relations of amino-acids to carbohydrates and the known conversion of ketonic acids into amino-acids suggest chemical possibilities which deserve consideration. From this point of view the protein of the leaf may represent the original protein from which all other kinds are formed, either directly or indirectly.

Up to the present time no recent publication dealing with the protein constituents of leaves has come to our attention. In the latter part of the 18th and the early part of the 19th century the fact that the green parts of living plants contain protein was recognized by several observers and to a small extent attempts were made to study this. Later when it was found that protein could be so much more easily isolated from seeds attention was diverted from the leaf.[1] Apart from numerous attempts to apply conventional methods for distinguishing non-protein nitrogen from protein nitrogen in leaves and green plants, little appears to have been done to increase our knowledge of this subject.

Botanists have made microscopic studies of leaves and other parts of plants which furnish some facts of interest respecting their protein constituents, but at present these reveal little respecting their chemistry or value in nutrition. We have not been able to find that any one has yet isolated the protein, as

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[1] For a discussion of this early history see Osborne, T. B., The vegetable proteins, Monograph on biochemistry, London, New York, Bombay, and Calcutta, 1909.

THE PROTEINS OF GREEN LEAVES.*

I. SPINACH LEAVES.

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INTRODUCTION.

For many years it has been the practice of agricultural chemists to state the proportion of protein which the cells of the green leaves contain. These proportions have been determined, however, by conventional methods and, apparently, in no case has any corresponding quantity of protein been isolated and identified by suitable chemical methods. The reason for our present meager knowledge of the protein constituents of living plants is chiefly due to the difficulties encountered in separating the contents of the cells from their enveloping walls. Attempts to grind the fresh leaf and extract the contents of the cells with water result in mixtures that cannot be filtered clear and consequently appear to present no opportunity to obtain the protein in a state fit for chemical examination. Attempts to extract dried leaves with water yield solutions containing only a part of the total nitrogen and most of this soluble nitrogen does not belong to protein. Such solvents as are usually used for extracting proteins from animal tissues, or from seeds, fail to dissolve much of the residual nitrogen.

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* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

such, from leaves in a condition suitable for studying its amino-acid make-up or its nutritive value.

The experience which we have had in the past in studying the proteins of seeds, and the improved methods now available for dealing with proteins, prompted us to make this investigation which finally showed how most of the protein can be easily separated from the fresh leaf.

Although preparations thus obtained doubtless are mixtures of several individual proteins they are more suitable for a study of the nutritive value of the total protein furnished by the leaf than would be any single protein contained in this mixture. We have consequently postponed attempts to make more detailed studies of them.

The Fresh Green Leaves.

Spinach leaves were employed for the present investigation because these contain more nitrogen than do most other leaves, and also because a fresh supply can be obtained throughout the greater part of the year. The fresh leaves consist of cells filled with cytoplasm, an apparently amorphous jelly-like substance, within which can be seen the nuclei and nucleoli of the cells, the chloroplasts, and also minute particles of unknown character. In addition the vacuoli and the water-conducting cells are filled with sap, a fluid containing substances doubtless in true solution. When the walls of the cell are broken by grinding, its contents are liberated and a mixture of the nuclei, chloroplasts, cytoplasm, sap, debris of cell walls, *etc.* is obtained. The parts contributing to this mixture are no longer in the same relation to one another as when intact within the living cell; they form an amorphous mixture, in which the components no longer can be recognized under the microscope. When this mixture is diluted with one or two volumes of water and either centrifuged at high speed or passed through coarse filter paper, an opaque green, slightly viscid fluid is obtained and also a residue, consisting chiefly of the cell walls. Although the fluid part is very opaque and looks as if it contained much suspended solids, microscopic examination reveals nothing except extremely minute granules and somewhat larger, spherical particles of insoluble ma [illegible] casionally can a fragment of the disintegrated chloroplasts be seen. Apparently the fluid passing the filter contains practically nothing except the soluble and the amorphous, colloidal constituents of the cell contents. If the grinding is thorough enough the filtered fluid may contain all the contents of the cells, while the solids retained by the paper are seen to consist only of the walls of the cells and the various ducts.

On a large scale it is impossible to grind the leaves so thoroughly that all the cells are disintegrated, but it is not difficult to grind them in a Nixtamal mill so thoroughly that the filtered solution will contain by far the greater part of the cell contents.

Addition of alcohol equivalent to one-fifth of this filtered mixture produces a bulky dark green precipitate which separates, leaving a green, slightly turbid solution. After separating this precipitate with the centrifuge the further addition of alcohol to one-third of the volume of the original extract yields another large green precipitate and a solution which can be easily filtered clear. On washing these green precipitates with strong alcohol chlorophyll and other substances are easily removed, leaving an almost colorless residue. When this is treated with ether a deep yellow extract is obtained which on evaporation leaves a semisolid fatty residue. The precipitates thus washed when air-dry are nearly colorless, friable products containing about 14 per cent of nitrogen, calculated ash-free.

A considerable part of the first precipitate is not soluble at room temperature either in an aqueous or in a 60 per cent alcoholic solution containing 0.2 to 0.3 per cent sodium hydroxide, although such solutions readily dissolve most types of proteins. If, however, the temperature of such an alcoholic solution is raised to boiling nearly all this precipitate dissolves. When the resulting solution is neutralized with acid most of the dissolved substance is precipitated and is then readily soluble at room temperature in a very slight excess of either acid or alkali. This behavior suggests that the protein was originally combined with other substances from which it is set free by hydrolysis with alkali. Proof of this, however, has not been obtained.

The second precipitate behaves like ordinary proteins, being almost completely soluble in cold dilute aqueous alkalies. Since in the original extract the protein in both the first and second precipitates apparently exists in colloidal rather than in true solution, for convenience we shall refer to it as the "colloidal protein."

At least 40 per cent of the total spinach nitrogen belongs to this colloidal protein; about one-third to substances which are soluble in water; and a comparatively small part of the other fourth of the nitrogen belongs to chlorophyll, phosphatides, *etc.*, soluble in strong alcohol; and presumably a part of the remainder to nucleic acid.

Since this colloidal protein is so readily precipitated by a relatively small proportion of alcohol while at the same time most of the chlorophyll, phosphatides, *etc.*, which were previously insoluble in pure ether, become soluble therein, it seems possible that within the cell these and possibly other substances exist in chemical union as a complex which forms a colloidal solution extremely sensitive to the action of alcohol. If this is so the solubility and other properties of the constituents of this complex give no idea of their chemical or physical properties as they are combined within the cell. We have long been familiar with similar combination of protein with lecithin in the yolk of hen's eggs. When these are mixed with a strong solution of sodium chloride and shaken with ether until freed from all that is soluble therein an aqueous solution is obtained which can be filtered easily. The clear filtrate yields an abundant precipitate of protein when salts are removed by dialysis and this precipitate dissolves *completely* in salt solution and can be reprecipitated and redissolved without giving any indication of the presence of phosphatides or fats. If, however, it is washed with alcohol more than 20 per cent of its dry weight is dissolved and this alcoholic solution contains phosphatides and fats which in our opinion were previously chemically combined with the protein. We suspect that similar combinations exist within the cell plasma of the leaf.

The residue of the cell walls, *etc.* contained about 16 per cent of the total nitrogen of the leaves, from which it seems fair to infer that about 84 per cent of the substance of the cell contents was mechanically removed by the method of disintegration described. Evidence was obtained that most of the nitrogen in this residue of the cell walls, *etc.* belonged to constituents of unruptured cells, or of those from which the contents had not been completely removed and was consequently protein nitrogen. As a result of this investigation it was found that about one-fourth of the solids of the spinach leaf is protein, and that one-fourth of its total nitrogen belongs to non-protein substances soluble in water.

The dry solids of the spinach leaf are much richer in protein than are any of the cereals and, in fact, some of the commercial protein concentrates such as bran, middlings, *etc.* If we remember that the cells of leaves are physiologically among the most active that we know it should not surprise us to discover that the chemical constitution of their contents is much like that of the cells of active animal tissues. The large proportion of water and cell walls makes the leaf appear to be a relatively poorer source of true nutrients than it actually is. If, however, we can learn to separate the contents of the cell from these we shall obtain a food product of very great value. This does not seem to be unattainable by means less complicated than those employed in flour milling. What may be the practical possibilities of concentrating and better utilizing the cell contents of leaves, such, for instance, as alfalfa, cannot be predicted, but the subject seems well worth considering.

EXPERIMENTAL.

1 kilo of the fresh leaves was ground by passing through a Nixtamal mill seven times. After adding 1,300 cc. of distilled water and passing through the mill three more times, most of the cells were ruptured and their contents suspended, or dissolved, in the fluid. From the results of analyzing a small aliquot of this mixture it was estimated that the ground leaves, which were treated in the way next described, contained 104.2 gm. of solids, dried at 107°, 6.0 gm. of nitrogen, and 19.3 gm. of ash. The mixture of ground leaves and water was centrifuged at high speed and the opaque, green fluid decanted from the coherent solids. These latter were washed by grinding again with 1,000 cc. of distilled water and centrifuged.

Precipitation with Alcohol.—After repeating this last treatment the three turbid solutions were each separately mixed with about one-fifth of their volume of 95 per cent alcohol. A large flocculent, dark green precipitate separated at once from the main extract, leaving a slightly turbid, green solution, but precipitates did not separate from the washings until a little sodium chloride was added. The precipitate from the second washings was small, showing that the extraction of the residue was nearly complete.

These precipitates, A, were separated by centrifuging, united, and washed with alcohol until the chlorophyll was removed. The alcoholic washings contained 4.4 gm. of solids, dried at 107°, 0.5 gm. of ash, and 0.1 gm. of nitrogen. The ash-free solids equalled 3.9 gm. Precipitate A was then washed with ether, which removed 0.8 gm. of a yellow semisolid grease, sparingly soluble in cold ether, but in boiling ether readily yielding a solution which gave an abundant precipitate when poured into acetone (lecithin ?).

Thus washed with alcohol and ether, Precipitate A was dried in the air at room temperature. It contained 0.5 gm. of ash, 1.1 gm. of nitrogen, and 0.034 gm. of phosphorus; its weight, ash- and moisture-free, was 7.9 gm.

More alcohol was added to the filtrates from Precipitate A produced by one-fifth volume of alcohol, until the volume was equal to one-third that of the original extract. This caused a second flocculent green precipitate, B, in each solution. These precipitates were united and washed with alcohol until the chlorophyll was removed. The alcoholic washings contained 2.2 gm. of solids, dried at 107°, 0.1 gm. of ash, and 0.04 gm. of nitrogen. The ash-free solids equalled 2.1 gm.

TABLE I.

	Precipitate A.	Precipitate B.
	per cent	*per cent*
Ash	7.4	0.2
Nitrogen	12.9 = 13.7 ash-free.	14.9 = 14.9 ash-free.
Phosphorus	0.4 = 0.4 "	0.3 = 0.3 "

Precipitate B was then washed with ether which extracted 0.5 gm. of a deep yellow grease. This appeared to contain more of the yellow oil and less of the colorless lecithin than did the ether extract of Precipitate A. Thus washed and dried in the air at room temperature, Precipitate B contained 0.03 gm. of ash, 1.6 gm. of nitrogen, and 0.022 gm. of phosphorus. The ash-free substance, dried at 107°, equalled 10.4 gm.

Since Precipitates A and B formed so much of the total solids of the leaf and appeared to consist mostly of protein they deserve special examination. The figures given in Table I facilitate a comparison of the data just given.

Probably Precipitate A contained more ash and less nitrogen than Precipitate B, because it carried down fine particles suspended in the turbid extract.

When hydrolyzed by boiling with 20 per cent hydrochloric acid for 14 hours these precipitates showed proportions of nitrogen in different forms as given in Table II.

The distribution of nitrogen among the several types is similar to that of most proteins with the exception of the humin nitrogen which is so very much greater as to suggest the presence of carbohydrate.

Examination of the Precipitates.—Although these figures indicate that Precipitates A and B consisted largely of protein they afford no satisfactory basis for an estimate of its actual proportion. Accordingly 3.48 gm. of Precipitate A and 4.67 gm. of Precipitate B were dissolved as far as possible by agitating with about 200 cc. of 0.2 per cent sodium hydroxide solution and removing the undissolved part by centrifuging. After repeating this extraction five successive times the clear alkaline solutions were united and neutralized with dilute hydrochloric acid. The precipitate which separated was washed with dilute alcohol, then with ether, and dried at 107°.

In the united filtrate and washings organic matter and nitrogen were determined. After removing alcohol, protein also was determined in this solution by saturating with ammonium sulfate. The difference between the total nitrogen and the ammonia nitrogen in the solution of the precipitate produced by the sulfate showed that 0.17 gm. of protein (N × 6.25) which had not been precipitated by neutralizing was present in this filtrate. The total organic solids in this filtrate weighed 1.5390 gm., hence at least 1.37 gm. or 16.8 per cent of non-protein organic matter must have been present in Precipitates A and B before treatment with the aqueous alkali. This filtrate contained 0.08 gm. of nitrogen of which 0.03 gm. was precipitated by saturating with ammonium sulfate from which it appears that non-protein nitrogenous matters also were present. A portion of this filtrate was hydrolyzed by boiling with 1 per cent hydrochloric acid for 2 hours and sugar estimated by reduction of cupric oxide. It was thus determined that the entire filtrate contained carbohydrate equal to 0.09 gm. of dextrose, or 0.10 gm. of xylose, or 0.08 gm. of xylan, from which it would appear that no considerable quantity of carbohydrate was present.

TABLE II.

	Precipitate A.	Precipitate B.
	per cent	*per cent*
Nitrogen as ammonia	7.9	7.2
Basic nitrogen	22.5	24.2
Humin "	7.5	5.0
Other " (by difference)	62.1	63.6
Total	100.0	100.0

The residues which failed to dissolve in the aqueous alkaline solutions were extracted three successive times by boiling for a few minutes with 60 per cent alcohol containing 0.3 per cent sodium hydroxide. The last extract yielded no precipitate when neutralized with acid. The two first clear extracts were neutralized with hydrochloric acid, and the precipitates washed first with dilute, then with strong alcohol, and finally with ether, and dried at 107°. Nitrogen and organic matter were determined in the precipitates and also in the filtrates and washings. The residues which failed to dissolve in the hot alkaline alcohol were washed with alcohol and ether and dried at 107°. Table III gives the results obtained.

These figures show that while 96 per cent of Precipitate B was soluble in aqueous alkali only 56 per cent of Precipitate A dissolved therein. Most of the insoluble part, however, did dissolve when boiled with the alkaline alcohol. Not all the nitrogen dissolved by the alkaline solutions was precipitated by neutralizing, for the filtrates contained nitrogen, a part of which was protein nitrogen precipitable by saturating with ammonium sulfate as has just been shown. While Precipitates A and B unquestionably contained some non-protein substance, 72 per cent of the ash-free substance of Precipitates A and B combined was recovered in the neutralization precipitates.

TABLE III.

			Precipitate A.	Precipitate B.
			gm.	*gm.*
Aqueous alkaline extract	Precipitate	Organic matter.....	4.571	
		Nitrogen...........	0.697	
	Filtrate....	Organic matter.....	1.539	
		Nitrogen...........	0.078	
Alcoholic " "	Precipitate	Organic matter.....	0.950	0.095
		Nitrogen...........	0.145	0.014
	Filtrate....	Organic matter.....	0.267	0.048
		Nitrogen...........	0.028	?
Insoluble residue.....................		Organic matter.....	0.293	0.053
		Nitrogen...........	0.012	0.002
		Ash................	0.091	?

The precipitate produced by neutralizing the united aqueous alkaline solutions of Precipitates A and B contained 1.47 per cent of ash, 15.25 per cent of nitrogen, and 0.09 per cent of phosphorus, calculated for the dry, ash-free substance. When hydrolyzed by boiling with 20 per cent hydrochloric acid for 14 hours proportions of the different forms of nitrogen were found as given in Table IV.

These figures agree quite closely with those found for Precipitate B and show that after solution in alkali and reprecipitation with acid the relatively low nitrogen content of this protein remains the same. This fact, together with the large amount of humin nitrogen, suggests that the protein may either be combined with, or contaminated by, carbohydrate or else with some group containing carbohydrate. A determination of pentose by distillation with hydrochloric acid gave a phloroglucin precipitate equal to 2.7 per cent of the protein as pentose or to 2.4 per cent as pentosan.

The uniform percentage of nitrogen found in the neutralization precipitates from the aqueous alkali, the alcoholic alkali, and, as will be shown later, from alkaline alcoholic extracts of another lot of dried spinach leaves (see p. 19), namely 15.25, 15.27, 15.52, and 15.41 respectively, indicates that some non-protein substance may be combined rather than admixed with the protein. A critical examination, however, of larger quantities of this protein will be needed to settle this question.

The Water-Soluble Constituents of the Leaves.—The united filtrates from the precipitate produced by one-third volume of alcohol measured 7,115 cc., a volume so large, in comparison with the amount of water-soluble constituents of the leaves, that there is little probability that any substances, actually soluble in water, were precipitated by the alcohol.

This solution was concentrated *in vacuo*, below 65°, to 2,650 cc. During concentration a very little substance separated which was not removed.

TABLE IV.

	Total N.	Protein.
	per cent	*per cent*
Nitrogen as ammonia	7.50	1.32
Basic nitrogen	22.49	3.96
Humin "	5.97	1.05
Other " (by difference)	64.04	8.92
Total	100.00	15.25

Determinations made on aliquots of 500 cc. showed that the entire solution contained 2.07 gm. of nitrogen, and, after deducting the sodium chloride which had been added, 45.57 gm. of solids containing 15.69 gm. of ash. The ash-free solids, therefore, were equal to 29.88 gm., and the total solids to 43.7 per cent of the leaves dried at 107°.

The remainder of this solution was saturated with ammonium sulfate, the precipitate treated with water, and the insoluble part removed by centrifuging, washed thoroughly with water, alcohol, and ether, and dried at 107°.

In terms of the entire water extract this insoluble fraction weighed 1.76 gm., and contained 0.227 gm. of nitrogen and 0.102 gm. of ash = 5.8 per cent of the fraction. The ash-free substance, which was almost completely soluble in dilute alkali and gave a good biuret reaction, weighed 1.66 gm. and contained nitrogen equal to 1.42 gm. of protein (N × 6.25) or to 85 per cent of the organic matter. Presumably this was mostly protein rendered insoluble in water by the treatment to which it had previously been subjected.

The solution of the ammonium sulfate precipitate was again saturated with this salt, and the precipitate freed as far as possible from sulfate by pressing between filter papers, and then dissolved in water. The difference between the total nitrogen and the ammonia nitrogen found in the solution, representing nitrogen of proteoses, was 0.286 gm., calculated for the whole water extract, or 1.79 gm. of proteose (N × 6.25).

The Insoluble Residue of the Leaves.—The microscope showed that the residue of the spinach leaves, which had been extracted three times with water, as already described, consisted chiefly of broken walls of cells and ducts. Among these were a few unruptured cells and some broken cells still retaining a part of their original contents. It was evident that the latter had been largely, though by no means completely, removed. This residue was then washed with alcohol and ether until chlorophyll was extracted. These washings contained 0.083 gm. of nitrogen, 3.212 gm. of solids, and 0.40 gm. of ash. The ash-free solids equalled 2.812 gm.

The washed residue, when dried at room temperature in the air, formed an almost perfectly white powder, equal to 32.25 gm., dried at 107°, and contained 0.960 gm. of nitrogen and 4.15 gm. of ash.

A quantity of this cell residue, equal to 9.243 gm., dried at 107°, was boiled with 500 cc. of 1 per cent hydrochloric acid for 4 hours and, after centrifuging, the residue was treated twice more in the same way. Pentosans and insoluble proteins were thus converted into soluble products by this mild hydrolysis. The solution contained 5.05 gm. of organic solids, 0.234 gm. of nitrogen, equal to 17.79 gm. and 0.81 gm. respectively in the entire residue. Hydrolysis had, therefore, rendered soluble 55.1 per cent of the solids and 85.0 per cent of the nitrogen. The part still undissolved, after washing with water and drying at 107°, weighed 2.636 gm. equal to 9.2 gm., calculated for the entire cell residue, or to 8.8 per cent of the spinach leaves. This residue contained 0.7 gm. of ash and 0.11 gm. of nitrogen equal to 0.67 gm. of protein, calculated for the original cell residue. Deducting these, the ash- and protein-free residue was equal to 7.5 per cent of the spinach leaves. This is decidedly more than the 5.5 per cent of crude fiber found in another sample by the conventional method of the agricultural chemists. The part undissolved by the boiling dilute acid also contained 0.031 gm. of nitrogen, equal to 0.108 gm. in the entire cell residue, or to 1.8 per cent of the nitrogen of the fresh spinach leaves.

The first acid extract, which contained 94 per cent of the total soluble solids and 92 per cent of the soluble nitrogen, was concentrated *in vacuo* to one-tenth of its volume. A considerable precipitate separated which partly dissolved on washing with water. The undissolved part, dried at 107°, weighed 0.747 gm. and consisted largely of inorganic matter. The soluble part reduced cupric oxide equivalent to 7.4 gm. of xylose calculated for the entire residue. If the second and third acid extracts contained the same proportion of xylose as did the first extract, the total xylose would be 7.5 gm., equal to 6.5 gm. of xylan.

Of the total nitrogen 19.4 per cent was free amino nitrogen and 4.9 per cent ammonia nitrogen. After boiling with 20 per cent hydrochloric acid for 10 hours the amino nitrogen was increased to 56.1 per cent of the total and the ammonia nitrogen to 7.4 per cent. These proportions are like those yielded by the colloidal protein and indicate that most of the nitrogen extracted from the cell residue by boiling with dilute acid was derived from this protein. Assuming this to be the case the total protein thus extracted would be equal to 5.10 gm. calculated for the entire cell residue. The protein and xylan thus estimated together are equal to 11.65 gm., leaving 6.14 gm. of organic substances of undetermined nature, or 5.9 per cent of the spinach leaves.

Table V summarizes the results of the preceding analysis of the fresh spinach leaves.

TABLE V.

	Solids ash-free.	Nitrogen.
	gm.	*gm.*
Soluble in alcohol and ether:		
Chlorophyll, lecithin, fat, *etc.*	10.1	0.24
Soluble in water:		
Proteose (N × 6.25)	1.8	0.29
Coagulable protein	1.4	0.23
Non-protein organic substances	26.7	1.56
In colloidal suspension in water	18.3	2.62
Extracted by boiling 1 per cent HCl	17.8	0.81
Residue of cell walls, *etc.*	9.2	0.11
	85.3	
Inorganic substances	19.3	
Total	104.6	5.86
Taken for analysis	104.2	6.02

We have shown that the precipitates produced by alcohol in the original turbid extracts consist chiefly of protein and, judging from their phosphorus, contain very little nucleic acid. We shall, therefore, not be far from the truth in estimating this protein by multiplying the nitrogen of these precipitates by 6.25. We have also shown that substances reducing copper oxide equal to 6.54 gm. of xylan and nitrogen, probably mostly of protein origin, equal to 5.1 gm. of protein are extracted from the residue of cell walls by boiling 1 per cent hydrochloric acid. The sum of the pentosan and protein, thus calculated, subtracted from that of the total organic solids of the acid extract gives 6.14 per cent of organic substances of still unknown character. The nature of the small amount of the nitrogenous substance remaining in the residue after extracting with boiling 1 per cent hydrochloric acid was not determined, but we have assumed that this was protein. Table VI gives the calculation of the results of this analysis in percentage of ash-free substances in the fresh spinach leaves.

These figures show that about one-fourth of the solids, as well as of the nitrogen, of the fresh spinach leaf belongs to non-protein, water-soluble substances, the nature of which has not yet been determined. Only a very small part of these contains the R-CONH$_2$ group because the ammonia, obtained by boiling the dried leaves with 60 per cent alcohol containing 0.3 per cent sodium hydroxide, amounted to only 1.6 per cent of the total nitrogen. About 17 per cent of the nitrogen in the water-soluble, non-protein substances is free amino nitrogen.

TABLE VI.

	Solids ash-free.	Nitrogen.
	per cent	*per cent*
Soluble in alcohol and ether:		
Chlorophyll, lecithin, fat, *etc*	9.7	4.0
Soluble in water:		
Proteose (N × 6.25)	1.7	4.8
Coagulable protein (N × 6.25)	1.4	3.8
Non-protein organic substances	25.6	25.9
In colloidal suspension in water:		
Protein (N × 6.25)	15.7	43.5
Non-protein substance (pentosans ?)	1.8	
Extracted by boiling 1 per cent HCl:		
Protein (N × 6.25)	4.9	13.6
Pentosans (xylan)	6.3	
Other organic substances	5.9	
Insoluble residue:		
Protein (N × 6.25)	0.6	1.7
Cellulose, *etc*	7.5	
	81.1	
Inorganic substances	18.5	
Total	99.6	97.3

How much of the remaining three-fourths of the nitrogen belongs to protein is not precisely established by the data here presented, but it is certain that the proportion of non-protein nitrogen is small. Most of the nitrogen of the Precipitates A and B, produced by alcohol as already shown, unquestionably belongs to protein. The small amount of phosphorus found in them limits the proportion of nucleic acid to not more than 3 or 4 per cent of these precipitates.

We have already given reasons for believing that most of the nitrogen in the cell residue belongs to protein. In so far as this nitrogen is derived from broken cells it is likely that most of the water-soluble protein escaped and passed into solution; and in so far as it is derived from unbroken cells very little can be assigned to water-soluble proteins because these contain so small a proportion of the total nitrogen of the leaf. Consequently we shall not be far from right in assuming that all this nitrogen belongs to the colloidal protein which had escaped extraction through failure to break up *all* the cells by grinding. The proportion of the different proteins in the fresh spinach leaves can be stated to be approximately as follows:

	Dry leaf. *per cent*
Proteose	1.7
Coagulable protein	1.4
Colloidal "	21.3
Total protein	24.4

From these figures it appears that the spinach leaf must be regarded as a food rich in protein.

The Dried Leaves.

Since many green foods, such as alfalfa or clover, consist chiefly of leaves and when dried and cured are fed as hay it seemed desirable therefore to examine the spinach leaves after they had been dried in a current of warm air below 60° and to compare the results obtained with those yielded by the green fresh leaves. Unfortunately, no supply of the lot of leaves used in the fresh state was available and it was necessary to use leaves which had been gathered early in the summer and were much less mature. As we intend later to apply the methods developed in this preliminary investigation to leaves of greater agricultural importance we have decided to publish the results which we obtained, because they show that drying at a low temperature has far less effect than one might expect on the nature or solubility of the constituents of the leaves.

The proportion of substances soluble in water is a little greater in the dried leaf than in the green, both protein and non-protein substances contributing to this small excess. The distinctly greater amount of proteose found in the extract of the dried leaf compared with the fresh indicates a slight autolysis during drying but proof that such a change actually occurs can only be secured by comparing the results obtained with parts of the same sample.

The percentage of the non-protein organic substances extracted from the dried leaves with water is the same as that remaining in solution after precipitating the turbid extract of the green leaves by the addition of one-third volume of alcohol. In both cases the percentage of the total nitrogen soluble in water is also alike. The amount of the colloidal protein obtained from these two sources was likewise nearly the same.

The results of this comparison justify the conclusion that data obtained with leaves carefully dried at a low temperature closely represent the composition of the leaf in its fresh, green condition.

EXPERIMENTAL.

The leaves, with about 1½ inches of stems attached, were carefully washed and dried in a current of warm air at a temperature not exceeding 60°. They were then ground to a very fine powder and successively extracted with ether, alcohol, and boiling water as follows:

Ether.—Two lots, I and II, of the air-dry spinach leaves were used. Each contained 18.22 gm. of solids, 0.866 gm. of nitrogen, and 4.44 gm. of ash, calculated for the leaves dried at 107°. Lot I was dried in the extraction capsule for 14 hours at 100°. It was covered with a plug of absorbent cotton, likewise dried, and extracted in the Soxhlet apparatus from which all moisture was removed by drying in the oven. The spinach leaves were then continuously extracted for 18 hours with ether which had been distilled over sodium. For comparison, Lot II in the air-dry condition was extracted with ordinary U.S.P. ether for the same time.

Alcohol.—Lot I was next extracted with absolute alcohol, Lot II with ordinary commercial alcohol, containing 6.5 per cent of water.

Boiling Water.—After thus removing everything soluble in ether, as well as in alcohol, Lots I and II were each extracted with about 400 cc. of boiling water five successive times, the residues being separated by centrifuging at high speed. The united extracts were concentrated to 500 cc. and nitrogen, solids, and ash determined in aliquots. The results of these extractions are given in Table VII.

The solids thus extracted by these several solvents were equal to percentages of the dried spinach leaves as shown in Table VIII.

The nitrogen extracted was equal to percentages of the total nitrogen of the dried spinach leaves as given in Table IX.

The spinach leaves contained 22.51 per cent of ash. The percentages of the total mineral matters extracted are given in Table X.

These figures show that these solvents extracted about one-half of the solids, one-fourth of the nitrogen, and nine-tenths of the mineral constituents.

Pure Ether.—Pure ether extracts nitrogen equivalent to 0.33 per cent of chlorophyll (N × 16.2) in the dry spinach leaves, or about 10 per cent

TABLE VII

	I. Pure ether.		II. U.S.P. ether.	
	gm.	*per cent*	*gm.*	*per cent*
Solids	0.5389	2.96	0.8545	4.70
Nitrogen	0.0036	0.02	0.0100	0.06
Ash	0.0093	0.06	0.0162	0.09
	I. Absolute alcohol.		II. 93.5 per cent alcohol.	
Solids	0.6255	3.43	1.0216	5.59
Nitrogen	0.0168	0.09	0.0486	0.21
Ash	0.0843	0.46	0.1851	1.01
	I. Boiling water.		II. Boiling water.	
Solids	8.1500	44.73	7.5940	41.51
Nitrogen	0.2190	1.20	0.1860	1.02
Ash	3.9375	21.61	3.7970	20.84

TABLE VIII.

Percentage of Solids Extracted from Dried Spinach Leaves by Various Successive Solvents.

Lot I.		Lot II.	
	per cent		*per cent*
Ether, pure	2.96	Ether, U.S.P.	4.70
100 per cent alcohol	3.43	93.5 per cent alcohol	5.59
Boiling water	44.73	Boiling water	41.51
Total	51.12		51.80

TABLE IX.

Percentage of the Total Nitrogen Extracted from Dried Spinach Leaves by Various Successive Solvents.

Lot I.		Lot II.	
	per cent		*per cent*
Ether, pure	0.40	Ether, U.S.P.	1.15
100 per cent alcohol	1.94	93.5 per cent alcohol	5.61
Water	25.29	Water	21.48
Total	27.63		28.24

of the solids soluble in pure, dry ether. Commercial U.S.P. ether extracted about three times as much nitrogen from the air-dry leaves as did dry ether. In terms of chlorophyll this would equal about 18 per cent of the solids extracted or 0.85 per cent of the leaves. Assuming that all this nitrogen belongs to chlorophyll, 2.63 and 3.85 per cent respectively would belong to other substances extracted by the two kinds of ether. It is evident that the alcohol and water in the U.S.P. ether has a marked effect on the solubility of these lipoids and but little on that of the inorganic matters.

Absolute Alcohol.—Absolute alcohol, after pure ether, extracted only 2.3 per cent of the spinach nitrogen. If this were all chlorophyll nitrogen about 1.5 per cent of the leaves, or about 43 per cent of the total solids of the alcoholic extract, might be chlorophyll. Since the solids extracted by absolute alcohol contained 13.5 per cent of mineral matter it is possible that, besides chlorophyll, salts or inorganic acids are also removed. The solids extracted by absolute alcohol dissolved readily in ether, although ether-soluble substances had previously been very completely removed. When this ether solution was poured into an excess of acetone a coherent deposit formed on standing, which, when treated with ether, was not completely soluble. On centrifugation a greasy sediment formed which, for the most part, was soluble in absolute alcohol.

TABLE X.

Percentage of the Total Mineral Ash Extracted from Dried Spinach Leaves by Various Successive Solvents.

Lot I.		Lot II.	
	per cent		*per cent*
Ether, pure	0.21	Ether, U.S.P.	0.36
100 per cent alcohol	1.90	93.5 per cent alcohol	4.17
Water	88.70	Water	85.52
Total	90.81		90.05

Apparently the excess of nitrogen removed by commercial alcohol belongs to substances soluble in boiling water, because the leaves previously extracted with pure ether and then with absolute alcohol, when exhausted with boiling water, yielded extracts containing a correspondingly greater amount of nitrogen. The excess of nitrogen extracted by commercial ether over that extracted by pure ether possibly belongs mostly to chlorophyll set free by the alcohol which the commercial ether contains.

Cold Water.—The residue remaining after extracting with ether, alcohol, and boiling water contained nearly three-fourths of the nitrogen of the leaf. Since this residue had been boiled with water it was not suitable for further examination of its proteins. Accordingly, after extracting dry spinach leaves with absolute ether and then with absolute alcohol, 20 gm.

of the dry residue (equal to 20.2 gm. of the original dry leaves and containing 0.95 gm. of nitrogen) were digested with 400 cc. of water at room temperature and then ground in a Nixtamal mill. After separating the solids by centrifuging, the residue was treated six more times in the same way. The last extract contained 0.2960 gm. of solids, dried at 107°, from which it is evident that it is practically impossible to remove completely all that is soluble in water.

The flocculent, insoluble matter that separated from the united extracts during concentration, when dried at 107°, weighed 0.6193 gm., and contained 0.0564 gm. of nitrogen and 0.0992 gm. of ash. This product contained 16.02 per cent of ash and only 10.85 per cent of nitrogen, calculated for the ash-free substance, equal to 5.94 per cent of the spinach nitrogen and to 0.3525 gm. of protein (N × 6.25) corresponding to 1.74 per cent of the dried spinach leaves.

The filtrate contained 8.984 gm. of solids, dried at 107°, equal to 44.47 per cent of the original dried leaves. The total solids thus extracted were equal to 47.53 per cent of the leaves. These contained 4.1820 gm. of ash and 0.308 gm. of nitrogen, equal to 32.42 per cent of the spinach nitrogen. Thus 38.36 per cent of the spinach nitrogen was soluble in water. The remainder of this filtrate was saturated with ammonium sulfate, the precipitate redissolved in water, and its solution again saturated with this salt. The precipitate was pressed on filter paper, dissolved in water, and ammonia nitrogen and total nitrogen were determined in the solution. By difference, 0.1075 gm. of nitrogen representing the nitrogen of proteoses was thus found. This corresponds to 11.32 per cent of the total nitrogen, or to proteose (N × 6.25), equal to 3.33 per cent of the original dried leaves. Deducting the nitrogen of the proteose from the total nitrogen of the extract leaves 21.1 per cent of water-soluble non-protein nitrogen in the spinach leaves.

Alkaline Solutions.—The residue of the leaves after extraction with cold water, when heated with either hot or cold dilute sodium hydroxide solution, yielded only a small part of its nitrogen to the extract. Cold 1 per cent hydrochloric acid also proved ineffectual. It was found, however, that by boiling for a short time with 60 per cent alcohol containing 0.3 per cent sodium hydroxide a relatively large amount of protein was extracted.

Accordingly spinach leaves, which had been dried in a current of warm air at 60°, were extracted with absolute ether and then with a mixture of ether and alcohol until freed from all that was soluble therein. These removed 7.8 per cent of solids. A quantity of the air-dry residue, thus extracted, equal to 9.951 gm. of the original leaves and containing 0.51 gm. of nitrogen was heated to boiling for about 5 minutes with 500 cc. of 60 per cent (by weight) alcohol containing 0.3 per cent of sodium hydroxide. The residue was washed twice by boiling with 400 to 500 cc. of 60 per cent alcohol. The extract and washings were made faintly acid to litmus with hydrochloric acid, concentrated *in vacuo* to about 400 cc., and then made clear by the cautious addition of very dilute sodium hydroxide solution.

Hydrochloric acid was then added until the precipitate separated as completely as possible. This was washed with 60 per cent alcohol, then with strong alcohol and ether, and dried at 107°. It weighed 1.78 gm. and contained 1.12 per cent of ash. The ash- and moisture-free substance weighed 1.77 gm., equal to 17.7 per cent of the original spinach leaves, and contained 0.274 gm. of nifrogen equal to 15.5 per cent of the precipitate or to 53.7 per cent of the total spinach nitrogen.

The filtrate contained 0.135 gm. of nitrogen, equivalent to 26.4 per cent of the spinach nitrogen and 0.008 gm. of nitrogen as ammonia, equal to only 1.5 per cent of the total nitrogen of the spinach leaves. The proportion of nitrogen in this filtrate was, therefore, approximately the same as that extracted by boiling water. The residue, after extraction with alkaline alcohol, weighed 4.64 gm., equal to 46.6 per cent of the original spinach leaves. It contained 0.077 gm. of nitrogen, equal to 15.1 per cent of the spinach nitrogen.

Since so large a proportion of the nitrogen remained in this residue another extraction was made using 60 per cent alcohol containing 0.4 per cent sodium hydroxide. The ash- and moisture-free protein precipitate obtained weighed 1.77 gm., equal to 17.8 per cent of the original dry spinach. This contained 0.274 gm. of nitrogen, equal to 15.4 per cent of the ash- and moisture-free protein or to 53.8 per cent of the spinach nitrogen. The filtrate from this precipitate contained 0.146 gm. of nitrogen equal to 28.5 per cent of the spinach nitrogen. The ammonia nitrogen was 0.008 gm., equal to 1.6 per cent of the spinach nitrogen.

Since an appreciable part of the ammonia might have been lost from the alkaline extract before this had been acidified we boiled the dried spinach leaves with 60 per cent alcohol containing 0.3 per cent sodium hydroxide and determined the total ammonia liberated, under conditions which precluded losses, to be the same as previously found. Since it is possible that a part of this ammonia originated from the non-protein, water-soluble nitrogenous substances which constitute about one-fourth of the total nitrogen of the spinach leaf it is probable that the protein lost little, if any, of its amide nitrogen during the short time it was heated with the alkaline alcohol. This conclusion is further supported by the fact that the nitrogen content of the protein thus extracted from the dry leaf was the same as that of the colloidal protein obtained from the fresh green leaf by means of cold dilute aqueous alkali.

The residue which had been extracted with alkaline alcohol, when dried at 107°, weighed 4.534 gm., equal to 45.5 per cent of the original leaves, and contained 0.068 gm. of nitrogen, equal to 13.3 per cent of the total nitrogen of the spinach leaves. The close agreement between the results of these extractions is shown by the figures in Table XI.

As these preparations contained the same amount of nitrogen as did the colloidal protein from the fresh leaves, and in other respects were so similar, it seems fair to presume that they are one and the same protein, or the same mixture of proteins. The conditions under which these preparations were extracted make it improbable that they contained any of the proteose and should they contain any of the coagulable protein the amount of this present in the spinach leaf is too small to contribute appreciably to the proportion of the nitrogen recovered in them. We, therefore, feel justified in concluding that at least one-half of the spinach nitrogen is contained in the colloidal protein. How much more of this escaped extraction it is impossible to say. The residue after extraction with alkaline alcohol contained 15 per cent of the spinach nitrogen, but, in view of the very thorough extraction, it is improbable that much of this was protein nitrogen, unless this was still retained within unruptured cells.

TABLE XI.

	60 per cent alcohol.			
	Plus 0.3 per cent NaOH.		Plus 0.4 per cent NaOH.	
	Per cent of solids.	Per cent of nitrogen.	Per cent of solids.	Per cent of nitrogen.
Protein	17.9	53.7	18.0	53.8
Filtrate	27.7	26.4	28.7	28.5
Residue	46.6	15.1	45.5	13.3
Ether solution	7.8	2.3	7.8	2.3
Total	100.0	97.5	100.0	97.9

DISCUSSION.

Comparison of the Results of Extracting the Fresh and Dried Spinach Leaves.

The proportion of solids as well as of nitrogen extracted from the dried leaves by ether, U.S.P., and 93 per cent alcohol and then by cold water is the same as that extracted from the fresh green leaves by the very different method described in the earlier part of this paper. This is shown in Table XII.

The water extract of the fresh leaves contained a little larger proportion of organic and a smaller proportion of inorganic solids than did that of the dry leaves (Table XIII).

The fresh leaves contained a much smaller percentage of mineral constituents than did the dried leaves which undoubtedly accounts for the difference in the inorganic substances shown by the figures in Table XIII.

The proportion of organic solids from the two sources is nearly the same. If, however, the ash-free solids of the ether-alcohol extracts are added the difference becomes not inconsiderable, namely 6.7 per cent, the total from the fresh leaves being 38.39 per cent and from the dry leaves 31.69 per cent. This excess of substance soluble in alcohol contained substances also soluble in water as we found that the residue left by evaporating off the alcohol nearly all dissolved in ether and that this solution left a residue largely soluble in water. No clue, however, has been obtained as to the nature of this water-soluble substance.

TABLE XII.

	Solids.		Nitrogen.	
	Fresh leaves.	Dry leaves.	Fresh leaves.	Dry leaves.
	per cent	*per cent*	*per cent*	*per cent*
Soluble in ether and alcohol...........	10.66	6.39	3.98	0.28
" " water, total solids..........	43.76	47.54	34.40	38.36
Total extracted.....................	54.42	53.93	38.38	38.64

TABLE XIII.

	Fresh leaves.	Dry leaves.
	per cent	*per cent*
Organic solids soluble in water.................	28.67	26.36
Inorganic " " " "	15.06	21.18
Total soluble solids.........................	43.73	47.54

During the early stages of the drying process, when the leaves are exposed to a temperature favorable for autolysis, changes of considerable magnitude may occur. Table XIV shows, however, that the proportion of the coagulable protein was only slightly greater and that of the proteose not very much greater in the extracts from the dry leaf than in those from the fresh, while the proportion of non-protein nitrogen was practically the same.

In how far these minor differences were due to autolytic changes occurring during drying or to differences in the composition of the two lots of leaves cannot be determined until new experiments are made.

We have shown that dilute alcohol precipitates 43.5 per cent of the spinach nitrogen from the colloidal solution obtained by grinding the fresh leaves with water, and that most of this is protein nitrogen. The residue of cell walls, *etc.* still contained nitrogen equal to 15.2 per cent of the total nitrogen of the leaf which for the most part is almost certainly protein nitrogen. As there is good reason to believe that most of this belongs to proteins of the same character as those found in the extract (see p. 12) we shall not go far wrong in assuming that in the fresh leaves at least 58 per cent of the spinach nitrogen, or 21.2 per cent of the spinach solids, belongs to this protein.

The protein extracted from the dried leaves by alkaline alcohol is unquestionably the same as that precipitated from the aqueous extracts of the fresh leaves by the addition of alcohol, and which we have provisionally designated as the colloidal protein. This protein from the dried leaves contained a little smaller proportion of the total spinach nitrogen than did the colloidal protein obtained from the water extract of the fresh leaves, but this deficit was made up by a correspondingly larger proportion of water-soluble proteins, possibly formed by autolysis during drying.

TABLE XIV.

	Solids.		Nitrogen.	
	Fresh leaves.	Dry leaves.	Fresh leaves.	Dry leaves.
	per cent	*per cent*	*per cent*	*per cent*
Proteose (N × 6.25)	1.7	3.3	4.8	11.3
Coagulable protein (N × 6.25)	1.4	1.7	3.8	5.9
Non-protein organic matter	28.7	26.4	25.9	26.4
Total	31.8	31.4	34.5	43.6

In estimating the approximate proportion of the colloidal protein in either the fresh or dry leaves we have not taken into account its possible contamination with insoluble non-protein nitrogenous compounds. Doubtless the nuclei of the cells contain nucleic acid, but either the amount of this is so small, or else its properties are so different from the nucleic acids with which we have been familiar, as to yield negative results with such tests as were employed. With the above reservations and assuming that these undetected non-protein nitrogenous substances contain too small a proportion of the spinach nitrogen to have importance for this preliminary investigation we can state the proportion of the spinach nitrogen among the various groups as given in Table XV.

TABLE XV

	Nitrogen.	
	Fresh leaves.	Dry leaves.
	per cent	*per cent*
Soluble in ether and alcohol	2.3	2.3
" " water:		
Non-protein substances	25.9	26.4
Proteose	4.8	11.3
Coagulable protein	3.8	5.9
Insoluble in water:		
Colloidal protein	58.7	53.8
Total	95.5	99.7

Stated in terms of percentage of the dry solids of the spinach leaf Table XVI shows the proportions of the different proteins (N × 6.25) obtained from the fresh and dry leaves respectively.

TABLE XVI.

	Fresh leaves.	Dry leaves.
	per cent	*per cent*
Proteose	1.7	3.3
Coagulable protein	1.4	1.7
Colloidal "	21.2	22.8
Total protein	24.3	27.8

It thus appears that protein substances contain approximately 67 per cent of the total nitrogen of the fresh leaves and 71 per cent of that of the dried leaves, or practically two-thirds of the nitrogen in each case. While these figures must be accepted as only roughly approximate we believe they afford a better basis for assuming that the greater part of the nitrogen of the leaf actually belongs to protein substances than has heretofore been presented. The conventional methods in common use have furnished support for such an assumption, but such indirect evidence as they afford has little value unless supported by chemical identification.

Concentration of the Spinach Protein.

By applying the results of this investigation to spinach leaves we have made a protein concentrate which we are now feeding to albino rats with marked success. This product was prepared by extracting the dried and finely ground leaves with boiling water whereby the large proportion of soluble salts and organic substances was removed. The loss of protein incurred by thus extracting was small since proteoses form but about 10 per cent of the proteins of this leaf. The dry residue, almost exactly one-half of the solids of the leaf, contained nitrogen equal to 39 per cent of protein (N × 6.25). We thus have as rich a protein concentrate as cottonseed meal.

If, as seems probable, similar products can be made from other green leaves it ought to be possible to feed these as the sole source of nitrogen and thereby increase our knowledge of the nutritive value of a class of proteins about which at present we know almost nothing. Possibly we shall also be able to learn something of the nutritive value of the water-soluble constituents. A new field for investigation appears to be thus opening which promises to be fruitful.

SUMMARY.

When fresh green spinach leaves are ground with water the contents of their cells are set free. The cell walls and other suspended matters can then be removed by filtering through soft paper or by centrifuging at high speed. A green turbid extract is thus obtained which contains the contents of the cells. The microscope shows that chloroplasts and all other formed elements have disappeared, only the most minute particles being visible, suspended in this fluid.

The addition of about one-third volume of alcohol to this extract causes a voluminous green precipitate to separate in large flocks leaving a clear solution.

The filtrate from this precipitate contains the water-soluble constituents of the cells, forming nearly one-half of the solids of the leaf. Only a small part of these is protein, proteoses being equal to only 1.7 per cent of the total solids of the leaf and proteins coagulable by heat to only 1.4 per cent. The non-protein organic substances are equal to about 28 per cent of the solids of the leaf and contain about one-fourth of its total nitrogen. The balance of these water-soluble constituents consists of inorganic matters.

The green precipitates produced by alcohol contain chlorophyll phosphatides, and fats which can be extracted by alcohol and ether, leaving a residue equal to about 20 per cent of the solids of the leaves and containing nearly 50 per cent of their nitrogen. This part of the alcohol precipitate consists almost wholly of protein, but, unlike other native proteins, it is not readily soluble in aqueous alkaline solutions at room temperature. When boiled for a few minutes with 60 per cent alcohol containing 0.3 per cent sodium hydroxide it dissolves and when this solution is neutralized with acid nearly all the protein is precipitated and is then readily soluble in a slight excess of either acid or alkali. This latter precipitate, which we have provisionally designated the colloidal protein, has the properties of pure protein, and contains 15.25 per cent of nitrogen. As it yields furfurol equivalent to 2.5 per cent of pentose this protein of the leaf may be a new type of combination of protein with carbohydrate-containing groups. However, it has not yet been established that the pentosan is actually combined, rather than admixed, with this protein.

Nucleic acid has not been detected in preparations of the colloidal protein. Their low phosphorus content shows that at the most the proportion of nucleic acid is small.

The colloidal protein is doubtless a mixture of several individual proteins which are constituents of the cytoplasm, protoplasts, and other elements of the cell, but owing to their insolubility in neutral solvents these cannot be separated from one another.

Apparently the colloidal protein occurs in the leaf, in chemical combination with chlorophyll, phosphatides, and probably other substances. This compound forms a colloidal solution with water which is very sensitive to the action of alcohol, being readily decomposed thereby into its component parts.

Spinach leaves dried at a low temperature and extracted with ether, alcohol, water, and alkaline solutions yield results so similar to those obtained with the fresh green leaf that evidently the constituents of the cells are altered to only a slight degree by drying.

DIGESTIBILITY OF RAW CORN, POTATO, AND WHEAT STARCHES.*

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INTRODUCTION.

It has been generally believed that raw starches are only digested a little, if at all, by the human body. Thorpe[1] quoting from Roberts says that starch in a raw state "is to man an almost indigestible substance, but when previously subjected to the operation of cooking it is digested with great facility." He further states that "diastase has, at best, only a comparatively feeble action on the unbroken starch granule, even at the temperature of the body."

Little experimental evidence is to be found regarding the digestibility of raw starches by the human body. Fofanow[2] conducted a series of experiments on human subjects with small quantities of raw wheat, oat, rice, and potato starches. He found that raw wheat, oat, and rice starches were practically completely assimilated, while raw potato starch was from two and one-half to four times less well digested. However, in his tests he used only 50 gm. of raw starch, a quantity which might easily be lost in the digestive tract. Numerous artificial digestion experiments have been conducted on raw starches which in general have demonstrated that raw starches are much more slowly acted on by the digestive ferments than starches which have been cooked.[3]

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*Published with permission of the Secretary of Agriculture.

[1] Thorpe, E., A dictionary of applied chemistry, London, New York, Bombay, and Calcutta, 1913, v, 179.

[2] Fofanow, L., *Z. klin. Med.*, 1911, lxxii, 257.

[3] Day, E. A., *U. S. Dept. Agric., Bull. 202*, 1908.

EXPERIMENTAL.

The methods in the experiments with corn, wheat, and potato starches were practically the same as in previous digestion experiments conducted by this office. In order that the starch should be in an appetizing form, it was eaten as a constituent of a frozen pudding. This was palatable and the subjects were able to eat it in fairly large quantities.

The frozen pudding contained approximately 20 per cent of raw starch and resembled ordinary ice-cream in taste and texture. This method of preparation did not affect the starch granules which were found by the Microchemical Laboratory of the Bureau of Chemistry to be neither swollen nor broken. The pudding was made as follows:

Experimental Frozen Pudding.

6 quarts milk.
4 pounds raw starch.
3 cups table oil.
2½ cups sugar.
1 tablespoon salt.
½ cup lemon extract.

The uncooked starch was mixed with milk, sugar, and oil in the proportions given above and the mixture immediately frozen in the same way as ice-cream. The lemon extract used for flavoring masked to a great extent the taste of uncooked starch and gave the frozen pudding a pleasing flavor.

The subjects were given weighed portions of the frozen pudding along with a basal ration of oranges and sugar. Tea or coffee was used if desired. The experiments were of 3 days or nine meals duration. The subjects were students in a local university who were apparently in normal health. They were familiar with this type of work, having served as subjects in previous experiments, and were entirely trustworthy. The methods for the separation of feces, analyses, *etc.* were those usually followed.

Corn-Starch.

Three experiments were conducted with raw corn-starch. The essential data for the interpretation of the results of these experiments are given in Tables I and II.

TABLE I.

Digestion Experiments with Uncooked Corn-Starch in a Simple Mixed Diet.

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1047, Subject W. V. D.:						
Frozen custard containing uncooked starch (= 67 per cent total carbohydrate = 644 gm.), *gm*.	3,365.0	2,030.1	76.4	277.3	961.4	19.8
Fruit, *gm*.	818.0	710.8	6.6	1.6	94.9	4.1
Sugar, *gm*.	138.0				138.0	
Total food consumed, *gm*.	4,321.0	2,740.9	83.0	278.9	1,194.3	23.9
Feces, *gm*.	76.0		21.6	30.5	10.2	13.7
Amount utilized, *gm*.			61.4	248.4	1,184.1	10.2
Digestibility of entire ration, *per cent*.			74.0	89.1	99.1	42.7
Estimated digestibility of uncooked starch alone, *per cent*.					100.0	
Experiment No. 1048 Subject H. L. G.:						
Frozen custard containing uncooked starch (= 67 per cent total carbohydrate = 773 gm.), *gm*.	4,038.0	2,436.1	91.7	332.7	1,153.7	23.8
Fruit, *gm*.	1,193.0	1,036.7	9.5	2.4	138.4	6.0
Sugar, *gm*.	62.0				62.0	
Total food consumed, *gm*.	5,293.0	3,472.8	101.2	335.1	1,354.1	29.8
Feces, *gm*.	55.0		15.8	19.7	10.8	8.7
Amount utilized, *gm*.			85.4	315.4	1,343.3	21.1

TABLE I—*Concluded.*

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Digestibility of entire ration, *per cent*			84.4	94.1	99.2	70.8
Estimated digestibility of uncooked starch alone, *per cent*					100.0	
Experiment No. 1049, Subject E. L.M.:						
Frozen custard containing uncooked starch (= 67 per cent total carbohydrate = 751 gm.), *gm.*	3,924.0	2,367.3	89.1	323.3	1,121.1	23.2
Fruit, *gm.*	1,127.0	979.4	9.0	2.3	130.7	5.6
Sugar, *gm.*	31.0				31.0	
Total food consumed, *gm.*	5,082.0	3,346.7	98.1	325.6	1,282.8	28.8
Feces, *gm.*	51.0		14.8	14.9	15.0	6.3
Amount utilized, *gm.*			83.3	310.7	1,267.8	22.5
Digestibility of entire ration, *per cent*			84.9	95.4	98.8	78.1
Estimated digestibility of uncooked starch alone, *per cent*					100.0	
Average food consumed per subject per day, *gm.*	1,633.0	1,062.3	31.4	104.4	425.7	9.2

The diet as a whole supplied on an average 31 gm. of protein, 104 gm. of fat, 426 gm. of carbohydrate, and had an average energy value of 2,760 calories per man per day. The average amount of raw corn-starch eaten per man per day was 241 gm. Subject H. L. G. in Experiment 1048 ate the maximum amount of raw corn-starch which was 258 gm. per day for the experimental period.

The coefficient of digestibility of the raw corn-starch was found in each case to be 100 per cent after correction is made for the undigested residue from the accessory foods. This was confirmed by the Microchemical Laboratory of the Bureau of Chemistry which reported that no unruptured starch grains could be detected in the feces and that the iodine test for starch in the feces was negative.

TABLE II.

Summary of Digestion Experiments with Uncooked Corn-Starch in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of uncooked corn-starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
1047	W. V. D.	74.0	89.1	99.1	42.7	100.0
1048	H. L. G.	84.4	94.1	99.2	70.8	100.0
1049	E. L. M.	84.9	95.4	98.8	78.1	100.0
Average		81.1	92.9	99.0	63.9	100.0

The ingestion of this frozen custard caused no noticeable physiological effects and the subjects were in normal health during the experimental period. They reported the diet as appetizing and satisfying, a fact which would indicate that the starch was assimilated to a great extent by the body.

Potato Starch.

Seven experiments were conducted with potato starch—two in one test and five in a second test a few weeks later. The two subjects who took part in the first test had taken part in the experiments with raw corn-starch. In the second test with raw potato starch these men were joined by three others, one of whom had taken part in the experiments with raw corn-starch.

Tables III and IV are a summary of the results of the experiments with raw potato starch.

The diet as a whole furnished on an average 23 gm. of protein, 76 gm. of fat, 357 gm. of carbohydrate, and had an average energy value of 2,213 calories per man per day. The average amount of starch eaten per man per day was 194 gm.

It will be noted from Tables III and IV that the amount of potato starch digested varied considerably with the different subjects and even with the same subject in successive periods. Subject J. F. S. in Experiment 1082 showed the highest coefficient of digestibility (95.2 per cent) while Subject E. L. M. in Experiment 1081 showed the lowest (62.3 per cent). Subject H. L. G. in the first test ate 573 gm. of raw starch of which 74.5 per cent was digested. In the second test the digestibility increased to 85.4 per cent despite the fact that 120 gm. more of potato starch were eaten than in the first. On the other hand, Subject E. L. M. in the first test ate 503 gm. of raw potato starch of which 74.3 per cent was digested while in the second test only 62.3 per cent of the 710 gm. eaten was digested. There seemed to be no definite relation between the amount eaten and the amount digested. A possible explanation of the variation in the ability of the various subjects and of the same subjects in different periods to digest the raw potato starch may be that the body was unable to supply a large enough amount of amylases in a given time to digest all the raw starch.

During this diet practically all the subjects noted a very excessive formation of gas and frequent intestinal cramps. The quantity of feces voided was very large; a large amount of undigested starch was visible and a strong positive reaction was given with iodine. When the feces were ashed, the odor resembled that of scorched bread and no fecal odor was evident. The figure for the digestibility of protein is probably too low, since only a small amount was eaten and no correction has been made for metabolic nitrogen in the feces. The coefficient of digestibility for the cream and corn oil was 95.7 per cent. This agrees closely with previous determinations by this office which were 96.9[4] per cent for cream and 97.9[5] per cent for corn oil.

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[4] Langworthy, C. F., and Holmes, A. D., *U. S. Dept. Agric., States Relations Service, Bull. 507*, 1917.

[5] Holmes, A. D., *U. S. Dept. Agric., States Relations Service, Bull. 687*, 1918.

TABLE III.

Digestion Experiments with Uncooked Potato Starch in a Simple Mixed Diet.

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1062, Subject H. L. G.:						
Frozen custard containing 572.6 gm. uncooked starch, *gm*.	2,823.0	1,707.8	60.7	216.4	816.5	21.6
Fruit, *gm*.	913.0	793.4	7.3	1.8	105.9	4.6
Sugar, *gm*.	68.0				68.0	
Total food consumed, *gm*.	3,804.0	2,501.2	68.0	218.2	990.4	26.2
Feces, *gm*.	194.0		17.3	6.1	163.0	7.6
Amount utilized, *gm*.			50.7	212.1	827.4	18.6
Digestibility of entire ration, *per cent*			74.6	97.2	83.4	71.0
Estimated digestibility of uncooked starch alone, *per cent*					74.5	
Experiment No. 1063, Subject E. L. M.:						
Frozen custard containing 502.8 gm. uncooked starch, *gm*.	2,491.0	1,508.5	53.7	191.5	718.3	19.0
Fruit, *gm*.	725.0	630.0	5.8	1.5	84.1	3.6
Sugar, *gm*.	105.0				105.0	
Total food consumed, *gm*.	3,321.0	2,138.5	59.5	193.0	907.4	22.6
Feces, *gm*.	191.0		27.8	10.7	143.9	8.6
Amount utilized, *gm*.			31.7	182.3	763.5	14.0
Digestibility of entire ration, *per cent*			53.3	94.5	84.1	61.9
Estimated digestibility of uncooked starch alone, *per cent*					74.3	

TABLE III—*Continued.*

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1078, Subject P. C.:						
Frozen custard containing 467.7 gm. uncooked starch, *gm.*	2,505.0	1,553.1	51.6	184.6	698.2	17.5
Fruit, *gm.*	1,372.0	1,192.3	11.0	2.7	159.1	6.9
Sugar, *gm.*	183.0				183.0	
Total food consumed, *gm.*	4,060.0	2,745.4	62.6	187.3	1,040.3	24.4
Feces, *gm.*	243.0		28.9	11.0	191.4	11.7
Amount utilized, *gm.*			33.7	176.3	848.9	12.7
Digestibility of entire ration, *per cent*			53.8	94.1	81.6	52.0
Estimated digestibility of uncooked starch alone, *per cent*					64.3	
Experiment No. 1079, Subject W. V. D.:						
Frozen custard containing 544.8 gm. uncooked starch, *gm.*	2,918.0	1,809.2	60.1	215.1	813.2	20.4
Fruit, *gm.*	807.0	701.3	6.5	1.6	93.6	4.0
Sugar, *gm.*	97.0				97.0	
Total food consumed, *gm.*	3,822.0	2,510.5	66.6	216.7	1,003.8	24.4
Feces, *gm.*	105.0		21.9	10.8	64.1	8.2
Amount utilized, *gm.*			44.7	205.9	939.7	16.2
Digestibility of entire ration, *per cent*			67.1	95.0	93.6	66.0
Estimated digestibility of uncooked starch alone, *per cent*					91.3	

TABLE III—*Continued.*

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1080, Subject H. L. G.:						
Frozen custard containing 691.3 gm. uncooked starch, *gm*........	3,703.0	2,295.9	76.3	272.9	1,032.0	25.9
Fruit, *gm*...........	731.0	635.2	5.8	1.5	84.8	3.7
Sugar, *gm*..........	43.0				43.0	
Total food consumed, *gm*......	4,477.0	2,931.1	82.1	274.4	1,159.8	29.6
Feces, *gm*............	149.0		18.2	7.3	117.4	6.1
Amount utilized, *gm.*			63.9	267.1	1,042.4	23.5
Digestibility of entire ration, *per cent*................			77.8	97.3	89.9	79.2
Estimated digestibility of uncooked starch alone, *per cent*................					85.4	
Experiment No. 1081, Subject E. L. M.:						
Frozen custard containing 710.0 gm. uncooked starch, *gm*........	3,803.0	2,357.8	78.3	280.3	1,059.9	26.7
Fruit, *gm*...........	526.0	457.1	4.2	1.1	61.0	2.6
Sugar, *gm*..........	105.0				105.0	
Total food consumed, *gm*......	4,434.0	2,814.9	82.5	281.4	1,225.9	29.3
Feces, *gm*...........	332.0		27.4	11.6	283.2	9.8
Amount utilized, *gm*..			55.1	269.8	942.7	19.5
Digestibility of entire ration, *per cent*................			66.8	95.9	76.9	66.6
Estimated digestibility of uncooked starch alone, *per cent*................					62.3	

TABLE III—*Concluded.*

Experiment, subject, and diet.	Weight of food.	Constituents of foods				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1082, Subject J. F. S.:						
Frozen custard containing 580.1 gm. uncooked starch, *gm*........	3,107.0	1,926.3	64.0	229.0	865.9	21.8
Fruit, *gm*..........	1,324.0	1,150.6	10.6	2.6	153.6	6.6
Sugar, *gm*..........	166.0				166.0	
Total food consumed, *gm*......	4,597.0	3,076.9	74.6	231.6	1,185.5	28.4
Feces, *gm*...........	100.0		24.3	8.9	52.1	14.7
Amount utilized, *gm*.			50.3	222.7	1,133.4	13.7
Digestibility of entire ration, *per cent*................			67.4	96.2	95.6	48.1
Estimated digestibility of uncooked starch alone, *per cent*...............					95.2	
Average food consumed per subject per day, *gm*..	1,357.9	891.4	23.6	76.3	357.8	8.8

TABLE IV.

Summary of Digestion Experiments with Uncooked Potato Starch in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of uncooked potato starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
1062	H. L. G.	74.6	97.2	83.4	71.0	74.5
1063	E. L. M.	53.3	94.5	84.1	61.9	74.3
1078	P. C.	53.8	94.1	81.6	52.0	64.3
1079	W. V. D.	67.1	95.0	93.6	66.0	91.3
1080	H. L. G.	77.8	97.3	89.9	79.2	85.4
1081	E. L. M.	66.8	95.9	76.9	66.6	62.3
1082	J. F. S.	67.4	96.2	95.6	48.1	95.2
Average...............		65.8	95.7	86.4	63.5	78.2

TABLE V.

Digestion Experiments with Uncooked Wheat Starch in a Simple Mixed Diet.

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1073, Subject P. C.:						
Frozen custard containing 462.4 gm. uncooked starch, *gm*........	2,493.0	1,573.3	48.4	164.8	690.3	16.2
Fruit, *gm*..........	728.0	632.6	5.8	1.5	84.5	3.6
Sugar, *gm*..........	120.0				120.0	
Total food consumed, *gm*......	3,341.0	2,205.9	54.2	166.3	894.8	19.8
Feces, *gm*............	31.0		5.1	5.7	16.5	3.7
Amount utilized, *gm*..			49.1	160.6	878.3	16.1
Digestibility of entire ration, *per cent*................			90.6	96.6	98.2	81.3
Estimated digestibility of uncooked starch alone, *per cent*...............					99.8	
Experiment No. 1075, Subject H. L. G.:						
Frozen custard containing 692.7 gm. uncooked starch, *gm*........	3,734.0	2,356.5	72.4	246.8	1,034.0	24.3
Fruit, *gm*...........	597.0	518.8	4.8	1.2	69.2	3.0
Sugar, *gm*..........	8.0				8.0	
Total food consumed, *gm*......	4,339.0	2,875.3	77.2	248.0	1,111.2	27.3
Feces, *gm*...........	56.0		4.5	17.9	23.4	10.2
Amount utilized, *gm*.			72.7	230.1	1,087.8	17.1
Digestibility of entire ration, *per cent*................			94.2	92.8	97.9	62.6
Estimated digestibility of uncooked starch alone, *per cent*...............					98.6	

TABLE V—*Concluded.*

Experiment, subject, and diet.	Weight of food.	Constituents of foods.				
		Water.	Protein.	Fat.	Carbohydrate.	Ash.
Experiment No. 1076, Subject E. L. M.:						
Frozen custard containing 597.9 gm. uncooked starch, *gm*........	3,223.0	2,034.0	62.5	213.0	892.5	21.0
Fruit, *gm*...........	1,294.0	1,124.5	10.3	2.6	150.1	6.5
Sugar, *gm*..........	201.0				201.0	
Total food consumed, *gm*......	4,718.0	3,158.5	72.8	215.6	1,243.6	27.5
Feces, *gm*............	68.0		6.1	17.6	33.5	10.8
Amount utilized, *gm*.			66.7	198.0	1,210.1	16.7
Digestibility of entire ration, *per cent*.			91.6	91.8	97.3	60.7
Estimated digestibility of uncooked starch alone, *per cent*...............					98.4	
Experiment No. 1077, Subject J. F. S.:						
Frozen custard containing 508.9 gm. uncooked starch, *gm*........	2,743.0	1,731.1	53.2	181.3	759.6	17.8
Fruit, *gm*...........	1,115.0	968.9	8.9	2.2	129.4	5.6
Sugar, *gm*..........	172.0				172.0	
Total food consumed, *gm*......	4,030.0	2,700.0	62.1	183.5	1,061.0	23.4
Feces, *gm*............	22.0		4.3	10.5	5.0	2.2
Amount utilized, *gm*.			57.8	173.0	1,056.0	21.2
Digestibility of entire ration, *per cent*.			93.1	94.3	99.5	90.6
Estimated digestibility of uncooked starch alone, *per cent*...............					100.0	
Average food consumed per subject per day, *gm*..	1,369.0	911.6	22.2	67.8	359.2	8.2

TABLE VI.

Summary of Digestion Experiments with Uncooked Wheat Starch in a Simple Mixed Diet.

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of uncooked wheat starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
1073	P. C.	90.6	96.6	98.2	81.3	99.8
1075	H. L. G.	94.2	92.8	97.9	62.6	98.6
1076	E. L. M.	91.6	91.8	97.3	60.7	98.4
1077	J. F. S.	93.1	94.3	99.5	90.6	100.0
Average		92.4	93.9	98.2	73.8	99.2

Wheat Starch.

Four experiments were conducted to determine the digestibility of raw wheat starch. All four of the subjects had taken part in the tests with raw potato starch, and two in the earlier ones with raw corn-starch.

The essential data for the interpretation of the experiments are given in Tables V and VI.

The diet as a whole furnished an average of 22 gm. of protein, 68 gm. of fat, and 359 gm. of carbohydrate per man per day with an average fuel value of 2,138 calories. The average amount of raw starch eaten per man per day was 188 gm. The subjects remained in apparently normal health during the diet period. It will be noted that practically all the wheat starch was assimilated in each case. No starch could be detected in the feces by the iodine test. Moreover, the other constituents were very well assimilated, the coefficients of digestibility being 92 per cent for protein and 94 per cent for fat.

SUMMARY.

1. Raw corn and wheat starches were found to be completely assimilated and no trace of them could be found in the feces.

2. Seven experiments on raw potato starch gave values for its digestibility varying from 62.3 to 95.2 per cent; the average was 78.2 per cent.

3. The ingestion of the potato starch caused disagreeable physiological disturbances not noted in the other experiments with raw corn and wheat starches.

4. The digestibility of the other constituents of the diet was not affected to any great extent by the large amounts of raw starch ingested.

THE CO_2 CONTENT AS A BASIS FOR DISTINGUISHING HEATED FROM UNHEATED MILK.

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INTRODUCTION.

It has been pointed out in a previous article[1] that the heating of milk, when pasteurized, reduces the CO_2 content to such an extent that this change might be made the basis of a method for distinguishing pasteurized from unheated milk. In order to ascertain whether this fact of decrease of CO_2 could be utilized in the development of such a method, it is necessary to learn to what extent the CO_2 content of normal milk is reduced under a variety of conditions, especially such conditions as are associated with the handling of milk from the time it leaves the udder until it reaches the consumer. A study has, therefore, been made of the effect of such conditions as (1) the method of milking, (2) the length of time of standing of milk after being drawn from the udder, (3) the effect of agitation, and (4) the effect of temperature. The results of this work are presented in the pages following.

The details of the method used in the determination of CO_2 in milk are explained in the article already referred to.

EXPERIMENTAL.

When milk is drawn from the udder and exposed to the air, a considerable proportion of its CO_2 escapes. The amount of CO_2 usually present in normal milk in the udder has been found in the former work, to which reference has already been made, to average about 10 per cent by volume. As a result of our work here given, we find that immediately after milk is drawn from the udder the CO_2 content is generally less than one-half of this figure, varying between 4 and 5 per cent in most cases.

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[1] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1919, xl, 335.

It is our purpose now to give the detailed results of our work, showing to what extent different conditions affect the CO_2 content of milk after leaving the udder.

1. *Effect of Method of Milking.*

A few determinations of CO_2 were made in the case of milk drawn from the udder by hand, in comparison with that drawn by milking-machine, two different kinds of machines being used. No appreciable difference was found in all the samples examined by us, the results varying only between 4 and 4.5 per cent of CO_2 by volume, without reference to the method of milking.

2. *Effect of Standing.*

Numerous examinations have been made by us to ascertain the amount of CO_2 in milk at different intervals after milking, when kept under ordinary conditions. Milk from individual cows and also from herds was used. After being drawn from the udder, the milk was kept under conditions involving a minimum amount of handling or agitation.

In the case of samples of herd milk, obtained at a local milk-station, the milk had been transported several miles directly from the farms to the station. The transportation was, of course, accompanied by more or less agitation. We examined forty such samples at intervals of 8, 20, and, in some cases, 40 hours after milking. The results are summarized in Table I.

These results indicate that, under the conditions existing, the volume per cent of CO_2 in no case falls below 3, even 40 hours after milking, and only in a few cases below 3.5 after 20 hours or more.

In the case of milk drawn separately from five individual cows in our station herd and examined, after standing without further handling, at intervals of 18, 42, 64, and 90 hours, the amount of CO_2 present in the milk soon after being drawn decreased by

0.5 to 1.0 per cent by volume in 18 hours, and 1.0 to 1.5 in 42 hours, after which there was no further decrease. The percentage of CO_2 in the fresh milk varied in the different samples from 4 to 4.5 and this dropped to a minimum of 3, no further decrease taking place after 42 hours.

In the case of the mixed milk of our station herd, which contained 4 per cent by volume of CO_2 3 or 4 hours after milking, it was found after standing 6 hours more to contain 3.5 per cent, a decrease of 0.5 per cent.

TABLE I.

CO_2 in Herd Milk at Intervals after Milking.

CO_2 in samples.	No. of samples examined after		
	8 hrs.	20 hrs.	40 hrs.
vol. per cent			
3.0	0	3	2
3.5	12	5	3
4.0	16	17	6
4.5	7	9	2
5.0	3	4	1
5.5	2	2	0

3. Effect of Agitation.

Several experiments were made to ascertain the effect of agitation in different forms upon the CO_2 content of milk. Milk was passed through a centrifugal separator in some cases and in others was stirred by special apparatus.

In the case of milk passed through a cream-separator, the milk before separation contained 4 per cent of CO_2 by volume. The resulting skim-milk was found to contain the same amount of CO_2, while the cream contained 3 per cent. In the case of another sample of milk, containing 3.5 per cent of CO_2 by volume before separation, the resulting skim-milk contained the same percentage of CO_2. Skim-milk containing 4 per cent of CO_2 by volume was found after being run through a separator to lose no CO_2.

In experiments in which the milk was stirred, the agitation was produced by means of the stirring-apparatus described in a former article.[2]

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[2] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

In the case of a sample of milk containing 4 per cent of CO_2 by volume, the milk was kept at room temperature (about 21°C.) during the stirring, and examinations were made at stated intervals, with the following results:

Length of time of stirring	0	15 min.	30 min.	45 min.	1 hr.	2 hrs.	6 hrs.
Percentage of CO_2 by volume	4	3.5	3	3	3	2.5	2

From these results it appears that such agitation as milk is subjected to in the methods commonly employed in handling milk on farms and before being placed on the market has little or no effect in reducing the percentage of CO_2.

4. Effect of Heating.

Experiments were made for the purpose of finding out the effect of increased temperature upon the CO_2 content of milk, using different temperatures, and also stirring at the same time in some cases. The special object in mind was to learn the effect of those conditions commonly employed in pasteurizing milk.

In the case of milk pasteurized by the "flash" system, samples containing 4 per cent of CO_2 by volume were found after pasteurization to contain not more than 2 per cent.

Numerous samples of market milk, pasteurized at about 62°C. (143°F.) and bottled, were examined 30 to 36 hours after pasteurization and found to contain 1.5 to 2 per cent of CO_2 by volume.

Milk heated at 62°C. (143°F.) for 30 minutes was examined before and after cooling to 5.5°C. (42°F.). The amount of CO_2 was found to be the same in both cases, 2 per cent.

Fresh milk containing 4 per cent of CO_2 by volume was heated at about 63°C. (145°F.) without agitation for different periods of time, the following results being obtained:

Minutes heated	2	3	4	5	10	12	20	30
Percentage of CO_2 by volume	3.5	3	2.5	2.5	2.5	2.5	2.5	2.5

A sample of milk heated to about 78°C. (172°F.), without stirring, for 30 minutes contained 2.5 per cent of CO_2 by volume. Another sample heated for 30 minutes to about 62°C. (143°F.) with stirring was found to contain no CO_2.

Milk, pasteurized by the methods commonly employed, and as found by us in the market, seldom contains more than 2.5 per cent of CO_2 by volume and usually contains less.

Distinction between Heated and Unheated Milk.

Taking into consideration all the results of our work, it appears that, under the conditions to which normal unheated milk is subjected in its handling from the time of milking to the time of delivery to the consumer, the volume percentage of CO_2 rarely, if ever, drops below 3 and seldom below 3.5; while the subjection of normal milk to the conditions of heating used in pasteurization reduces the percentage of CO_2 by volume to 2.5 or less. Therefore, it appears safe, in general, to assume that milk containing less than 2.5 or 3 per cent of CO_2 by volume has been heated to the temperature of pasteurization.

A STUDY OF THE DISTRIBUTION OF IODINE BETWEEN CELLS AND COLLOID IN THE THYROID GLAND.

I. METHODS AND RESULTS OF STUDY OF BEEF, SHEEP, AND PIG THYROID GLANDS.

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(From the Laboratory of Physiological Chemistry and Pharmacology, University of Chicago, Chicago.)

(Received for publication, March 8, 1920.)

At the present time there seems fairly general agreement among investigators that iodine in the thyroid gland plays a significant rôle in thyroid function. As a matter of fact most biochemical and physiological studies of thyroid gland activity have centered about iodine which has been taken as the basis of potency of preparations and a presumptive index of the state of the gland.

Morphological studies have partially correlated the architectural characteristics with iodine content of the gland. Oswald,[1] Kocher,[2] Marine and Williams,[3] and others have found a fair parallelism between the quantity of colloid and the quantity of iodine of approximately normal glands. It must be remembered, however, that the estimation of the amount and concentration of colloid in any specimen of gland has not as yet been placed on a satisfactory basis, consequently the concentration of iodine in the colloid material cannot be accurately judged. On the other hand more evidently extreme colloid and hyperplastic types of glands appear to be anomalous in this relation which according to Kocher[2] is due to variations in concentration of colloid material.

Concerning the relative significance of colloid to glandular activity Bensley[4] brought out morphological evidences based on selective staining reactions indicating that the active secretion of the thyroid gland passes through what is usually considered the bases of the cells into blood or lymphatics and that the visible colloid represents either a waste product or a reserve. Whether colloid is considered as a reserve or as a waste product the mode of its quantitative diminution or access to the circulation is yet an unsolved problem.

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[1] Oswald, A., *Virchows Arch. path. Anat.*, 1902, clxix, 444.

[2] Kocher, A., *Virchows Arch. path. Anat.*, 1912, ccviii, 86.

[3] Marine, D., and Williams, W. W., *Arch. Int. Med.*, 1908, i, 349.

[4] Bensley, R. R., *Am. J. Anat.*, 1916, xix, 37.

One great difficulty in evaluating the relative importance and significance of cells *versus* colloid, the latter being usually considered the result of active secretion of the gland, has been the inability to locate and follow accurately the course of the iodine-containing body within the gland itself. Most investigators believe, following Baumann[5] and Oswald,[6] that the colloid is a secretion and is the material in which iodine is to be found. Oswald,[6] it will be recalled, scraped out mechanically colloid material from colloid-rich glands fixed in 70 per cent alcohol and found iodine. The iodine content of the cells of colloid-containing glands had not been specifically determined.[7]

As a consequence, it is highly desirable to apply a method adequate to locate the iodine within the gland in order to determine whether it is all in the colloid, all in the cells, or whether there exists a physiological equilibrium between the iodine of the colloid and the iodine of the cells, and further if there exists such an equilibrium it is desirable to determine the controlling factors. It is by such a study that evidences may be obtained bearing on the physical chemistry of the gland and the relative significance of colloid and active cells. Further, such knowledge might likely have an immediate bearing upon our conceptions of the mode of secretion and character of action of the normal stimulants to the gland whatever they may subsequently be found to be. It is with this basic problem of localization of iodine-containing constituents in the thyroid gland that the work here presented is concerned.

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[5] Baumann, E., *Z. physiol. Chem.*, 1895–96, xxi, 319; 1896–97, xxii, 1.

[6] Oswald, A., *Z. physiol. Chem.*, 1899, xxvii, 14.

[7] Claude, H., and Blanchetière, A., *J. physiol. et path. gén.*, 1910, xii, 563.

Methods.

Separation of Cells from Colloid.

As is perhaps generally known to morphologists frozen section preparations of unfixed thyroid glands do not retain the colloid material when such sections are floated in isotonic salt solution. It is evident then that in such preparations the fluid colloid readily leaves the opened acini. I have repeatedly tried to obtain evidences of colloid by selective staining reactions in such sections and have always failed. Furthermore when the frozen preparations were prepared in an ammonia-cooled room, the completely frozen section being placed immediately upon a slide, it was observed that after bringing the slide outside the cold chamber into a room at ordinary room temperature the colloid was readily demonstrated to have spread more or less uniformly over the whole section as would glycerol or other fluid. These facts led to the idea of utilizing such preparations as a means of separating cells from colloid for purposes of chemical analysis.

Thyroid glands obtained from the abattoir[8] or the laboratory as soon as possible after death of the animals were cut into blocks of suitable size for cutting on the freezing microtome. Sections were cut as thin as efficiently possible, or at least sufficiently thin to open all acini. Such sections were placed in Ringer's solution immediately from the microtome knife, the sections separated from one another, and straightened out. After a sufficient quantity of sectioned material was obtained the sections were either picked out with a dissecting needle or, as a routine procedure, the whole mixture was centrifuged directly. The liquor was poured off (in some instances this liquor itself was analyzed and found to contain iodine), then a second portion of salt solution added to the cell mass, and the whole thoroughly stirred in order to remove any adhering colloid material. This second mixture was centrifuged, the liquor poured off, and then the cell mass, including unavoidable stroma, or connective tissue, washed out with a small quantity of distilled water onto a watch-glass. The material was evaporated carefully over a hot plate and then finally dried at 105°C. Unsectioned specimens of the whole gland were dried in the same manner for control values.

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[8] Through the courtesy of Armour and Company, Chicago.

TABLE I.

Quantitative Determination of Iodine in Whole Glands and of Cells Free from Colloid Material.

Animal No.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of percentage of iodine in cells to percentage of iodine in whole gland.	Morphology.	
						Cells.	Colloid.
	mg.	*per cent*	*mg.*	*per cent*			
Pig 1	250.2	0.377	171.5	0.076	0.20	Flat.	Rich.
“ 2	222.6	0.81	127.2	0.274	0.34	“	“
Sheep 1	251.0	0.26	104.0	0.096	0.37	“	“
“ 2	367.0	0.442	126.5	0.145	0.33	“	“
“ 3	266.5	0.265	125.5	0.076	0.28	“	“
“ 4	297.0	0.198	139.5	0.046	0.23	“	“
“ 5	364.0	0.19	111.0	0.078	0.41	“	“
“ 6	334.0	0.273	112.0	0.086	0.31	“	“
“ 7	258.5	0.165	93.0	0.065	0.39	“	“
“ 8	308.5	0.089	195.0	0.027	0.30	Cuboidal.	Fair.
“ 9	231.0	0.129	90.3	0.048	0.37	Flat.	Rich.
Beef 1	288.0	0.151	177.0	0.128	0.85*	“	“
“ 2	248.0	0.025	155.5	0.011	0.44	Columnar.	Poor.
“ 3	184.0	0.308	153.7	0.128	0.42	Flat.	Rich.
“ 4	166.0	0.468	145.0	0.192	0.41	“	“
“ 5	141.0	0.409	163.0	0.118	0.29	“	“
“ 6	263.0	0.429	148.0	0.118	0.28	“	“
“ 7	145.0	0.021	111.5	None.		Columnar.	Poor.
“ 8	228.5	0.103	80.5	0.054	0.53*	“	“
“ 9	240.5	0.065	186.5	0.055	0.86*	“	“
“ 10	134.5	0.023	118.3	0.011	0.48	“	“
“ 11	200.5	0.043	182.5	0.009	0.21	“	“
“ 12	207.0	0.042	113.5	0.015	0.36	“	“
“ 13	252.2	0.039	249.5	0.014	0.37	“	“
“ 14	202.8	0.366	165.5	0.111	0.30	Flat.	Rich.
“ 15	248.9	0.040	215.5	0.010	0.25	Columnar.	Poor.
“ 16	343.5	0.051	220.8	0.020	0.39	“	“
“ 17	349.3	0.005	319.3	0.0054	1.08†	“	“
“ 18	302.0	0.095	191.5	0.041	0.43	“	“

* Unexplainable high values.

† Cells appear to contain a higher concentration of iodine than whole gland, though the actual concentration is too low to be accurately determinable in such amounts of whole gland and of cell material used.

Iodine Determination.

The method used for iodine determination was that described by Kendall.[9] This method was checked prior to adoption, by analysis of dried thyroid gland previously analyzed by the Hunter method, and was found to be satisfactory for such material containing measurable quantities of iodine or quantities of material containing as a minimum 0.008 mg. of iodine. The limits of sensitivity appear to be rather the limits of thiosulfate titration than the preparatory technique.

The actual concentration of iodine in thyroid cells is evidently higher than the values given in Table I for the reason that not alone epithelial cells but also connective tissues within and about the gland are included in the cell mass examined for iodine. However, the same criticism applies as well to the whole gland for the iodine concentration is based on total gross weight of the material analyzed.

DISCUSSION.

It will be seen from Table I that, whereas the individual percentages of iodine in different whole glands vary, the cells, with the exception of those from one gland, all contain measurable amounts of iodine, and furthermore that the *ratio of the percentage of iodine in cells to percentage of iodine in the whole gland comes to a fairly constant value.*

It is true that within the series of ratios themselves as much as 100 per cent variation occurs, yet they are nevertheless of the same order of magnitude and hence may be considered a physiological constant for glands in this particular functional condition. The values of the ratios appear to fall well within relatively narrow limits, regardless of the percentage values of the iodine in whole glands which differ among themselves up to 1,000 per cent. The wide variation of total iodine values adds considerable weight to the evidence of a relative percentage constant. While in the table of twenty-nine analyzed glands there are four examples in which high ratios were obtained, it does not seem likely that the remaining twenty-four fall between the limits of 0.20 and 0.48 by mere coincidence but are rather of some basic significance. An occasional high value may be expected since in the method of separation adopted it is possible to have some unopened acini or incomplete separation or other unavoidable errors in technique. We feel that the lower values are more likely to be more exact. All available data have been presented without reservation, so that the case presented may stand on its own merits.

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[9] Kendall, E. C., *J. Biol. Chem.*, 1914, xix, 251.

The observed relative percentage constant may be quite analogous to the partition coefficient of a substance distributed between the solvents in a diphasic system. On the other hand this same percentage constant need not necessarily hold for pathological states within the gland which may conceivably alter one or both phases or even the solute itself (iodine-containing molecule). Seasonal variation, various stages in physiological activity, or any other unknown factors might possibly alter the ratio though such does not appear to be evident from the series presented. These problems, however, are now being further investigated and will be reported in subsequent papers of this series of studies.

In regard to the dog thyroid glands, previously reported in abstract,[10] it may be stated that by using as a criterion the ratio values found for other species, the iodine values of the cells of such amounts of glands available from one animal calculate close to or below the lowest limits of sensitivity of the method for iodine determination. This explanation will rectify the conclusion recorded in the preliminary notice in which there did not appear to be any measureable iodine in the cell fraction. It is only rational then to suppose that by taking glands from several animals that a sufficient quantity of cells could be obtained so that quantitative studies of iodine content could be accurately made. This question will be more fully reported in a subsequent paper. It may be now stated, however, that by taking a sufficient quantity of cell material iodine is usually to be found though in very low concentration.[11]

It is desirable to add that the conclusions drawn herewith depend upon the safety of the method. As to the question of the effects of freezing upon diffusibility of iodine-containing compounds, whether or not under such a procedure the iodized molecule more readily leaves the cells or on the other hand enters the cells, the following evidences may be cited. Subsequent fixation and staining are inadequate to demonstrate any readily detectable alterations in morphology. Concerning diffusion changes it has been found that cells from iodine-poor glands placed in colloid-containing liquor obtained from iodine-rich glands do not appear to alter in iodine content within an hour or so at room temperature.[11] It must be admitted, however, that the iodine concentration of the liquor (Ringer's solution plus dissolved colloid) is far lower than the original colloid bathing the cells *in situ*. Yet this seems to us to answer possible objections on the basis of our measurements being a question of mere equilibria between cells in suspension and iodine of the colloid in solution. Since the liquor was relatively rich in organically bound iodine, cells poor in iodine should on a purely physical basis have been raised in iodine content to a value equal to that of the cells from the gland rich in iodine. But such was not the case. The iodine content of cells from the iodine-poor gland remained the same whether placed in iodine-rich or iodine-poor liquor.

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[10] Tatum, A. L., *Proc. Soc. Exp. Biol. and Med.*, 1919, xvii, 28.

[11] From part of the work now in progress in this laboratory in collaboration with Mr. H. B. Van Dyke.

SUMMARY.

1. A new method is described for the separation of cells from colloid of the thyroid gland for chemical study.
2. Iodine is found in both cells and colloid of beef, sheep,and pig thyroid glands.
3. The ratio of percentages of iodine in cells to iodine in whole glands appears to be relatively constant for pig, sheep, and beef thyroid glands, even though such glands vary both in morphology and in total iodine content.

FURTHER STUDIES ON THE ELIMINATION OF TAURINE ADMINISTERED TO MAN.*

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(Received for publication, March 3, 1920.)

The question as to the fate of certain amino-acids when given in large doses has been the subject of numerous papers. The evidence that the portion which is excreted by the kidney is combined with urea in the form of uramino-acid rests on the basis that this compound has been isolated from urine.

Schultzen [1] isolated sarcosincarbamic acid from the urine of a dog fed with sarcosin, but the quantitative differences in yield noted by Baumann and von Mering,[2] Salkowski,[3] and Schiffer [4] on repeating this experiment can hardly be attributed to individual variability of the experimental animals. The ease with which amino-acids combine with urea to form the corresponding uramino-acid [5] would lead one to suspect that the latter when found in urine was not preformed in the body but a resultant of the process used for its isolation, and the quantity obtained dependent upon the particular technique used. Dakin[6] fed inactive tyrosine and phenylalanine to cats and although he found, in accordance with results previously obtained by Blendermann,[7] uramino-acids in the urines of his experimental animals he recognizes the possibility that these substances were formed in the process of isolation from the unchanged amino-acid present in the urine. When the urine was acidified with acetic acid as soon as passed the yield of uramino-acid was so small as to be insufficient for identifica-

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* Aided in part by a grant from the George Williams Hooper Foundation for Medical Research.

[1] Schultzen, O., *Ber. chem. Ges.*, 1872, v, 578.

[2] Baumann, E., and von Mering, J., *Ber. chem. Ges.*, 1875, viii, 584.

[3] Salkowski, E., *Z. physiol. Chem.*, 1880, iv, 100.

[4] Schiffer, J., *Z. physiol. Chem.*, 1881, v, 257.

[5] Weiland, W., *Biochem. Z.*, 1912, xxxviii, 385. Lippich, F., *Ber. chem., Ges.* 1908, xli, 2974.

[6] Dakin, H. D., *J. Biol. Chem.*, 1910, viii, 25, 35.

[7] Blendermann, H., *Z. physiol. Chem.*, 1882, vi, 234.

tion. The failure of Rohde[8] to obtain by analytical methods, after decomposing the urea with urease, evidence of the presence of uramino-acid in the urine of a cat injected with phenylalanine, indicates that the α-ureidio-β-phenylpropionic acid isolated by Dakin[9] in a similar experiment was formed after the urine had been passed.

The experiments of Salkowski[10] on the elimination of taurine have been of particular interest to us. He found that taurine when taken by mouth was eliminated in large part in the urine. This was determined by estimating the increase in neutral sulfur. The substance which he isolated was not taurine but taurocarbamic acid. Experiments reported by Schmidt, von Adelung, and Watson[11] confirmed Salkowski's findings with regard to the increase of neutral sulfur. It, however, appears to us in view of the doubt which exists as to the formation of uramino-acids in the body that the taurocarbamic acid isolated by Salkowski was formed after the urine had been passed. Our data presented below show that taurine is excreted in the free state and not combined with urea as taurocarbamic acid.

Since taurine yields its nitrogen quantitatively in 4 minutes[12] when shaken with nitrous acid while the uramino-acid gives off its nitrogen but slowly, use was made of the method of Van Slyke[13] for the determination of α-amino nitrogen and the nitrogen due to slowly reacting amines in the urine of a subject to whom taurine was administered. If taurine is excreted in the urine in the free state an increase in α-amino nitrogen over the normal output should be noted, but if it is present as taurocarbamic acid the nitrogen obtained from the slowly reacting amines should show a corresponding increase. Preliminary experiments in which taurine and taurocarbamic acid were added to urine showed that taurocarbamic acid was not split by urease,[14] and that the added taurine could be quantitatively estimated from the increase of α-amino nitrogen.

A subject was placed on a constant diet and the normal excretion of total nitrogen, amino-acid nitrogen, and sulfur determined.

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[8] Rohde, A., *J. Biol. Chem.*, 1918, xxxvi, 467.

[9] Dakin, H. D., *J. Biol. Chem.*, 1909, vi, 235.

[10] Salkowski, E., *Virchows Arch. path. Anat.*, 1873, lviii, 460, 580; *Ber. chem. Ges.*, 1872, v, 637.

[11] Schmidt, C. L. A., von Adelung, E., and Watson, T., *J. Biol. Chem.*, 1918, xxxiii, 501.

[12] Foster, M. G., and Hooper, C. W., *J. Biol. Chem.*, 1919, xxxviii, 355.

[13] Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 125.

[14] Previously noted by Mateer, J. G., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1916, xxv, 297.

TABLE I.

Subject I.

Day of experiment.	Total nitrogen.	α-amino nitrogen.	Nitrogen from slowly reacting amines.*	Total sulfur.	Total sulfates.	Neutral sulfur.	Remarks.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
1	10.1	0.38	0.11	0.80	0.66	0.14	
2	10.3	0.44	0.05	0.82	0.67	0.15	
3	9.3	0.38	0.10	0.72	0.58	0.14	
4	11.0	1.12	0.12	2.49	0.70	1.79	10 gm. taurine by mouth. S = 2.56 gm.
5	9.8	0.46	0.09	0.84	0.63	0.21	
6	9.3	0.38	0.09	0.78	0.56	0.22	

* Calculated on the basis of 4 minutes.

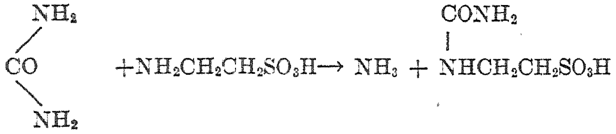
TABLE II.

Subject II.

Day of experiment.	Total nitrogen.	Urea nitrogen.	Urea nitrogen in total nitrogen.	Ammonia nitrogen.	Ammonia nitrogen in total nitrogen.	Remarks.
			Experiment A.			
	gm.	*gm.*	*per cent*	*gm.*	*per cent*	
1	9.0	7.4	82	0.58	6.4	
2	9.4	7.4	79	0.54	5.7	
3	8.7	6.7	77	0.66	7.6	3.3 gm. taurine by mouth in 10 (hourly) doses.
4	9.4	7.3	78	0.57	6.1	
5	10.4	7.3	70	0.79	7.6	14 gm. taurine by mouth in 10 (hourly) doses.
6	9.3	7.4	80	0.65	7.0	
			Experiment B.			
1	6.6	5.7	86	0.44	6.7	
2	7.5	6.2	83	0.52	6.9	
3	8.7	7.0	80	0.51	5.9	
4	9.5	7.0	74	0.48	5.1	15 gm. taurine intravenously.
5	8.2	7.2	88	0.43	5.2	
6	8.8	6.8	77	0.46	5.2	15 gm. taurine intravenously.
7	8.1	6.4	79	0.56	6.9	

He was then given 10 gm. of taurine by mouth and similar determinations were carried out for this day as well as for several succeeding days. The results are given in Table I. It is at once evident that ingestion of taurine has resulted in an increase of both neutral sulfur and α-amino nitrogen, in amounts which correspond to about 63 per cent of the ingested taurine. There is no increase in the slowly reacting amines, indicating that no appreciable amount of taurine has combined with urea to form taurocarbamic acid. On the basis of increase in total nitrogen excreted, the amount of taurine eliminated is greater than that calculated from the sulfur or α-amino nitrogen. Since there is a greater variability in the total nitrogen figures the latter method of calculation seems the more accurate.

Several other experiments were carried out to determine the effect, if any, of the ingestion of taurine on the excretion of urea and ammonia in urine (Table II). If urea and taurine combined *in vivo* and the reaction follows the same course as *in vitro*



$$CO(NH_2)_2 + NH_2CH_2CH_2SO_3H \rightarrow NH_3 + \underset{}{\overset{CONH_2}{N}}HCH_2CH_2SO_3H$$

a decrease in urea and a corresponding increase in ammonia nitrogen should be expected. Within the normal variability the amounts of urea and ammonia excreted are not affected by the administration of taurine. These figures support those of the previous experiment and point to the conclusion that taurocarbamic acid is not a metabolic end-product when taurine is ingested.

SUMMARY.

1. Determinations of neutral sulfur and amino-acid nitrogen in the urine of an individual given 10 gm. of taurine by mouth show a close agreement in the increase of α-amino nitrogen and neutral sulfur over the control period, indicating that taurine is not eliminated as taurocarbamic acid.

2. Administration of taurine in large doses does not result in any marked changes in the excretion of urea and ammonia.

SOME PROTEINS FROM THE GEORGIA VELVET BEAN, STIZOLOBIUM DEERINGIANUM.

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(Received for publication, March 8, 1920.)

The Georgia velvet bean is a sport of the Florida velvet bean, *Stizolobium deeringianum*, from which it differs principally in having the shortest season of all the cultivated *Stizolobia*, so that it can be successfully grown in the extreme northern part of the cotton belt. It is used chiefly as cattle food and in 1917 its production had already increased to such an extent as to exceed that of any other plant of the group. The beans themselves are of a mottled color, dark brown and white. They are spherical to ovoid in shape, slightly flattened, about $\frac{3}{8}$ inch in greatest diameter, and extremely hard and tough.

An examination of the proteins of the closely related Chinese velvet bean, *Stizolobium niveum*, has already been published by this laboratory.[1] The increasing importance of the Georgia strain, however, seemed to make it desirable that the latter also should be made the subject of a special study.[2] Considerable differences in the nature of the protein content of the two seeds have been brought out, as will be seen if comparison is made of this with the preceding paper.

The Georgia velvet bean contains about 23.6 per cent of protein (N × 6.25). 3 per cent sodium chloride solutions extract a maximum of about 15 per cent of protein from the finely ground seed; and a yield of 13 per cent of mixed proteins is obtainable by coagulation from the slightly acidified extract at the boiling temperature.

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[1] Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, 1918, xxxiv, 429.

[2] For information concerning the development, culture, and use of velvet beans we are indebted to Dr. C. V. Piper of the Bureau of Plant Industry of the Department of Agriculture. The beans used in this investigation were furnished by the Bureau of Plant Industry.

From the sodium chloride extracts we have separated three types of protein: a globulin preparation, referred to in this paper as the α-globulin, which is precipitated from the extract by 0.4 of saturation with ammonium sulfate, coagulates at 70–78°C., and contains 0.90 per cent of sulfur; a second type of globulin preparation, which we have designated the β-globulin, requiring 0.6 to 0.7 of saturation with ammonium sulfate for precipitation, coagulating at 90–100°C., and containing but 0.45 per cent of sulfur; and an albumin type, obtained by coagulation from aqueous extracts previously freed from globulin by prolonged dialysis. The globulins are thus precipitated, and are removed by decantation and filtration, while the albumin remains in solution. This coagulates at 54–62°C., and contains about 1.00 per cent of sulfur.

TABLE I.

Percentages of the Basic Amino-Acids in the Proteins from the Georgia Velvet Bean.

Amino-acid.	α-Globulin.		β-Globulin.		Albumin.
	I	II	I	II	I
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Cystine............	1.03	1.04	0.91	0.87	1.92
Arginine............	7.25	7.14	8.09	8.28	6.13
Histidine...........	1.34	1.14	3.32	3.43	0.82
Lysine.............	8.20	8.44	8.61	8.42	8.20

Particularly striking is the behavior of these preparations in the qualitative test for *tryptophane* by Hopkins and Cole's reaction. The α-globulin shows strongly the color reaction characteristic of tryptophane; but the β-globulin gives no trace of the reaction, even after long standing. The albumin preparations give the test at once, and after several hours standing the color becomes very intense.

The percentages of the basic amino-acids in these proteins, as determined by Van Slyke's method,[3] are given in Table I.

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[3] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15.

EXPERIMENTAL.

Preliminary.

Extraction Experiments.—The whole beans were crushed in a power-driven meat chopper and ground to a fine meal in a pulverizing mill. Extractions were then made to ascertain the solvent best suited for the extraction of the proteins. 10 gm. samples of the meal were thoroughly stirred with 200 cc. portions of the solvent. The mixtures were allowed to stand for 24 hours at room temperature in order to secure as complete an extraction as possible. The supernatant liquid was then filtered off through small folded filters, and nitrogen determined in 20 cc. aliquots. The results (Table II) indicate 3 per cent salt solution as the most effective of the solvents tried. This solution was used for the extraction of the globulin preparations described in this paper.

TABLE II.

Preliminary Extraction Experiments.

Solvent (NaCl).	Nitrogen extracted from meal.*	Protein extracted from meal.*
per cent	*per cent*	*per cent* ($N \times 6.25$)
10	1.75	10.94
7	2.04	12.73
5	2.30	14.41
3	2.42	15.09
1	2.23	13.95
0.1	1.66	10.36

* Computated as percentage of total dry substance.

Precipitation with Ammonium Sulfate.—To determine the behavior of the proteins in the saline extract toward ammonium sulfate, 10 cc. portions of the extract were made successively from 0.1 to 0.9 saturated with the precipitant, filtering after each addition through a small folded filter moistened with an ammonium sulfate solution of the same concentration as that of the test solution. These tests indicated the presence of but *one* globulin fraction, that precipitated by 0.4 of saturation with ammonium sulfate; and a number of preparations were made. It was later observed, however, in working with large volumes of extract (5 or 6 liters), that a small further precipitate was produced by increasing the concentration of ammonium sulfate to 0.6 of saturation. Separations of the two globulin fractions were then made as described in the following section.

The α- and β-Globulins.

Extraction.—2 or 3 kilos of meal were thoroughly stirred with five volumes of a 3 per cent distilled water solution of pure sodium chloride. The extraction was allowed to continue for 24 hours at about 2°C. Filter paper scrap was then added in sufficient quantity to produce a pulp of a consistency suitable for pressing, the pulp placed in muslin bags, and the liquid pressed out in a powerful press. The resulting turbid extract was filtered by suction through thick mats of filter paper pulp on Buchner funnels and the filters were washed once with a small volume of 3 per cent salt solution.

Preparation of the α-Globulin.—The filtered extract was measured and made 0.4 saturated with ammonium sulfate. The finely powdered precipitant was added slowly and with vigorous stirring, each portion being completely dissolved before more was added. The voluminous precipitate became flocculent and settled in about ½ hour. It was filtered off on a folded filter, washed from the filter with 0.4 saturated ammonium sulfate solution, again filtered off, and finally redissolved with distilled water, the ammonium sulfate which remained in the paste supplying in this case the neutral salt necessary for the solution of the globulin. This solution, after filtering clear, was dialyzed 10 or 12 days in parchment paper bags against running cold water. The precipitate thus formed was washed free from remaining traces of chlorides with distilled water, dehydrated by suspension for 24 hours in absolute alcohol, freed from alcohol by washing on a Buchner funnel with absolute ether, and dried by gradually heating to 110°C. in a vacuum oven. Preparations made in this manner constituted the α-globulin. The yield is from 2.75 to 3.00 per cent of the meal extracted.

Preparation of the β-Globulin.—The filtrate and washings from the original precipitate of α-globulin were measured and made 0.5 saturated with ammonium sulfate and the extremely small precipitate was removed. From the filtrate the β-globulin was thrown out by adding ammonium sulfate up to 0.7 of saturation. Most of this fraction is thrown down at 0.6 of saturation with the precipitant, and separation is complete at less than 0.7 of saturation. The purification and drying of this material were carried out exactly as described for the α-globulin. The yield is about 1.25 per cent of the meal extracted.

Physical Properties.—The precipitation limits of the globulins with ammonium sulfate have been noted in the preceding paragraphs. The coagulation temperatures were determined by redissolving small portions of the dialysis precipitates in 3 per cent salt solution, adding 0.5 per cent of acetic acid, and heating at the rate of about 0.5°C. per minute until coagulation began and then very slowly until the precipitate was flocculent and the supernatant liquid clear. The apparatus used was similar to that employed in the determination of melting points, except that a test-tube containing the solution under examination with a thermometer immersed therein was substituted for the thermometer with attached melting point tube. Under these conditions the α-globulin shows cloudiness at 70°C., a flocculent precipitate at 74–75°C., and apparently complete coagulation with the supernatant liquid clear at 78°C. The β-globulin clouds at about 90°C., shows a flocculent appearance at 97°C., and the cloudiness of the supernatant liquid disappears but slowly at the boiling temperature.

No evidence of crystalline form could be observed in the dialysis precipitates of either of the globulins. The appearance of the α-globulin under the microscope was that of extremely minute, amorphous, translucent particles; the β-globulin was in all respects similar, except that the particles were considerably coarser. The finished preparations are pale gray, dusty powders, light, and decidedly hygroscopic; exposed to the air, they absorb from 6 to 10 per cent of moisture.

Qualitative Examination.—The ordinary protein reactions are shown by both globulins, with the exception of the reaction of Hopkins and Cole, which depends upon the presence of *tryptophane.* The color characteristic of this amino-acid develops immediately, and strongly, when the test is applied to the α-globulin, but careful and repeated testing of all our preparations of the β-globulin failed to show any trace of the reaction.[4] This is particularly interesting inasmuch as no vegetable globulin has hitherto been shown to lack tryptophane. The *prolamin,* zein, from *Zea mays* is the only vegetable protein which heretofore has been shown to be entirely without this amino-acid.

Molisch's test indicates the presence of but traces of carbohydrate in the α-preparations, since the color given is very faint. The β-preparations, with one exception, did not show the reaction at all; in the one case in which the test was positive the color was barely detectable. The faint colors produced in this very sensitive test would seem to indicate that the traces found are impurities merely, probably filter paper fibers.

Phosphorus was not found in either protein.

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[4] Osborne and Mendel have been kind enough to test this β-globulin for us, and have confirmed our results.

TABLE III.

α-Globulin.

	Preparation I.				Preparation II.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon.....	52.49	52.68	52.59	53.03	52.80	52.89	52.85	52.97
Hydrogen...	6.78	6.69	6.74	6.80	6.77	6.81	6.79	6.81
Nitrogen....	16.58	16.62	16.60	16.74	16.72	16.72	16.72	16.76
Sulfur......	0.92	0.86	0.89	0.90	0.91		0.91	0.91
Oxygen*....				22.53				22.53
Ash........	0.79		0.79		0.24		0.24	
	Preparation III.				Preparation IV.			
Carbon.....	53.09	53.30	53.20	53.36	52.39	52.45	52.42	52.97
Hydrogen...	6.79	6.92	6.86	6.88	6.71	6.72	6.71	6.79
Nitrogen....	16.66	16.69	16.68	16.73	16.20	16.28	16.24	16.41
Sulfur......	0.93		0.93	0.93	0.88		0.88	0.89
Oxygen*....				22.10				22.94
Ash........	0.32		0.32		0.93		0.93	

* By difference.

TABLE IV.

β-Globulin.

	Preparation I.				Preparation II.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon.....					52.85	52.87	52.86	53.16
Hydrogen...					6.77	6.76	6.77	6.80
Nitrogen....					17.09	16.93	17.01	17.11
Sulfur......	0.44	0.45	0.45	0.45	0.45		0.45	0.46
Oxygen*....								22.47
Ash.........	0.63		0.63		0.55		0.55	
	Preparation III.				Preparation IV.			
Carbon.....	53.29	53.28	53.29	53.34	52.66	52.69	52.68	52.99
Hydrogen...	6.71	6.78	6.75	6.76	6.75	6.79	6.77	6.81
Nitrogen....	17.18	17.07	17.13	17.17	17.54	17.35	17.45	17.55
Sulfur......	0.45		0.45	0.46	0.44		0.44	0.45
Oxygen*....				22.26				22.20
Ash.........	0.11		0.11		0.58		0.58	

* By difference.

Elementary Composition.—On account of their hygroscopic properties which render the accurate weighing of dry samples impossible, all preparations were exposed in thin layers until equilibrium with the moisture of the air was reached. Moisture and ash were then determined, and all analyses calculated on the moisture-free basis. The results are given in Tables III and IV.

TABLE V.

*Distribution of Nitrogen in the α-Globulin as Determined by Van Slyke's Method.**

Sample I, moisture- and ash-free, 3.0212 gm. protein, 0.5057 gm. nitrogen.†
" II, " " " 2.9788 " " 0.4984 " "

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0457	0.0445	9.04	8.93	8.99
Humin N adsorbed by lime	0.0077	0.0064	1.53	1.30	1.42
" N in amyl alcohol extract	0.0002	0.0015	0.04	0.30	0.17
Cystine N	0.0037	0.0036	0.73	0.72	0.73
Arginine N	0.0696	0.0695	13.75	13.95	13.85
Histidine N	0.0093	0.0108	1.85	2.17	2.01
Lysine N	0.0490	0.0469	9.69	9.42	9.56
Amino N of filtrate	0.2994	0.2952	59.20	59.23	59.22
Non-amino N of filtrate	0.0218	0.0210	4.32	4.22	4.27
Total nitrogen recovered	0.5064	0.4994	100.15	100.24	100.22

* Nitrogen figures corrected for the solubilities of the bases.

† Nitrogen content of protein, 16.73 per cent.

TABLE VI.

Basic Amino-Acids in the α-Globulin.

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	1.03	1.04	1.04
Arginine	7.25	7.14	7.20
Histidine	1.34	1.14	1.24
Lysine	8.20	8.44	8.32

Distribution of Nitrogen in the Globulins as Determined by Van Slyke's Method.—Duplicate samples, about 3 gm., of each of the globulins were hydrolyzed by boiling for 24 hours with 100 cc. of 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method.[5] The results are given in Tables V, VI, VII, and VIII.

TABLE VII.

*Distribution of Nitrogen in the β-Globulin as Determined by Van Slyke's Method.**

Sample I, moisture- and ash-free, 2.8713 gm. protein, 0.4918 gm. nitrogen.†
" II, " " " 2.8708 " " 0.4917 " "

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0532	0.0536	10.81	10.89	10.85
Humin N adsorbed by lime	0.0070	0.0074	1.41	1.50	1.46
" N in amyl alcohol	0.0002	0.0002	0.04	0.04	0.04
Cystine N	0.0031	0.0029	0.63	0.59	0.61
Arginine N	0.0748	0.0766	15.21	15.57	15.39
Histidine N	0.0259	0.0267	5.26	5.43	5.34
Lysine N	0.0475	0.0464	9.65	9.44	9.55
Amino N of filtrate	0.2580	0.2572	52.46	52.30	52.38
Non-amino N of filtrate	0.0229	0.0192	4.65	3.90	4.28
Total nitrogen recovered	0.4926	0.4902	100.12	99.66	99.89

* Nitrogen figures corrected for the solubilities of the bases.

† Nitrogen content of protein, 17.13 per cent.

TABLE VIII.

Basic Amino-Acids in the β-Globulin.

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	0.91	0.87	0.89
Arginine	8.09	8.28	8.19
Histidine	3.32	3.43	3.38
Lysine	8.61	8.42	8.52

The Albumin.

Preparation.—1 or 2 kilos of meal were stirred with five volumes of distilled water and the extraction was allowed to continue over night at about 2°C. The mixture was then prepared and pressed and the extract filtered, exactly as in the preparation of the globulins. The extract was dialyzed directly, without previous treatment with ammonium sulfate, for 10 or 12 days and the precipitated globulin removed by decantation and filtration. The clear filtrate was dialyzed again for several days, the precipitate removed, and the dialysis resumed. This process was continued till the precipitation of the globulins appeared to be complete. The albumin was obtained from the filtered dialysis liquors by coagulation at a temperature which was not allowed to exceed 65°C. (the coagulation range is from 54–62°C.); this coagulum, after washing with warm distilled water until the last washings were free from chlorides, was dried by means of absolute alcohol and ether with final heating to 110°C. *in vacuo*, as was done in the case of the globulin preparations. The yield is 0.6 to 0.75 per cent.

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[5] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

Physical Properties.—The albumin coagulates from its slightly acidified solution within the range 54–62°C. The finished preparations are very similar in appearance to those of the globulins, though somewhat lighter in color.

Qualitative Examination.—The ordinary protein reactions are given by this material. Hopkins and Cole's color reaction for tryptophane develops slowly but becomes very strongly positive on standing. Traces of carbohydrate are present; probably the feebly positive test is due to minute quantities of filter paper fibers.

Elementary Composition.—The same procedure was adopted as in the analysis of the globulins, the results being calculated on the moisture-free basis. The albumin preparations are sharply differentiated from those of either of the globulins by their higher sulfur and lower nitrogen content. The analysis is summarized in Table IX.

Nitrogen Distribution in the Albumin as Determined by the Van Slyke Method.—Owing to the extremely small yield of this protein, material for duplicate determinations was not obtained. 2.0079 gm. of the albumin, equivalent to 1.8327 gm. of moisture- and ash-free protein, were hydrolyzed by boiling with 100 cc. of 20 per cent hydrochloric acid for 24 hours. Analysis of the hydrolysate was then made according to Van Slyke's directions, using the amyl alcohol-ether method[5] for the decomposition of the precipitate of the basic amino-acids. The calculated nitrogen of the sample is 0.2931 gm.; recovered, 0.2933 gm. The figures obtained are given in Tables X and XI.

TABLE IX.

Albumin.

	Preparation I.				Preparation II.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbon......	52.61	52.76	52.69	53.36	49.51	49.49	49.50	53.43
Hydrogen...	6.72	6.67	6.70	6.80	6.27	6.20	6.24	6.73
Nitrogen....	15.67	15.81	15.74	15.96	15.05		15.05	15.76
Sulfur......	0.99	1.01	1.00	1.02	0.97		0.97	1.00
Oxygen*....				22.86				23.08
Ash.........	1.23		1.23		3.75		3.75	

* By difference.

TABLE X.

*Distribution of Nitrogen in the Albumin as Determined by Van Slyke's Method.**

Sample, ash- and moisture-free, 1.8327 gm. protein, 0.2931 gm. nitrogen.†

	gm.	*per cent*
Amide N..	0.0266	9.08
Humin N adsorbed by lime.....................	0.0053	1.80
" N in amyl alcohol extract...............	0.0000	0.00
Cystine N...	0.0041	1.41
Arginine N..	0.0362	12.35
Histidine N.......................................	0.0041	1.41
Lysine N..	0.0289	9.85
Amino N of the filtrate.........................	0.1881	64.25
Non-amino N of the filtrate....................	0.0010	0.34
Total nitrogen regained.......................	0.2943	100.49

* Nitrogen figures corrected for the solubilities of the bases.
† Nitrogen content of protein, 15.99 per cent.

TABLE XI.

Basic Amino-Acids in the Albumin.

	per cent
Cystine..	1.92
Arginine...	6.13
Histidine..	0.82
Lysine...	8.20

SUMMARY.

The Georgia velvet bean contains 23.6 per cent of protein (N×6.25). Experiments with sodium chloride in various concentrations indicate a 3 per cent aqueous solution as the most effective solvent. This solution extracts about 15 per cent of protein.

From the 3 per cent sodium chloride extracts a yield of about 13 per cent of mixed proteins may be secured by coagulation with heat. By the fractional precipitation of the extract with ammonium sulfate, however, and by subsequent purification of the fractions as described in this paper two globulins, designated as the α- and β-globulins, may be separated, in yields of about 3 and 1.25 per cent, respectively; and an albumin is found in extracts which have been freed from globulin by repeated dialysis. The latter protein may be coagulated in yields of about 0.75 per cent by the prolonged heating of its slightly acidified solution at 65°C.

Analyses of the three proteins show marked differences in the nitrogen and sulfur content, and in the distribution of nitrogen as determined by Van Slyke's method.

Qualitative examination indicates that the β-globulin contains no tryptophane, an observation of particular interest in view of the fact that this amino-acid has been found to be present in all seed globulins heretofore tested. The α-globulin and the albumin from the Georgia velvet bean both contain tryptophane.

EXPERIMENTAL STUDIES ON GROWTH.*

XV. ON THE GROWTH OF RELATIVELY LONG LIVED COMPARED WITH THAT OF RELATIVELY SHORT LIVED ANIMALS.

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Experimental Results.

In previous articles of this series[1] experiments have been described in which the growth of white mice fed upon a varied and abundant diet, to which specified unusual additions had been made, was compared with the growth of similar animals fed upon the same diet but without the unusual additions. Since these experiments and observations were continued without interruption from shortly after the birth of the animals until the occurrence of death from natural causes, they offer the opportunity of instituting a highly important comparison, which has not heretofore been attempted; namely, the comparison at stated brief intervals of the growth in weight of animals which survive to an age in excess of the average duration of life with that of animals in which death antedates the average duration of life. Since individual records of the weights of all the animals were kept throughout, and deaths from essentially accidental causes, such as epidemic infections, were reduced by our technique to a minimum[2] this comparison could be made by simply sorting the animals of each experimental group into two subgroups, the one,

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* The expenses of this research were in part defrayed by a grant from the Special Medical Research Fund of the University of Toronto.

[1] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347; 1919, xxxvii, 377, 393, 427, 455. Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 363, 385, 397; xxv, 635, 647.

[2] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347; 1919, xxxvii, 443.

the short lived group, of animals dying of causes other than accidental injury or epidemic infection before the average duration of life for the group under consideration, and the other, the long lived group, of animals dying subsequently to the average duration of life. The weights of each of the subgroups are averaged week by week until 7 months of age and fortnightly thereafter. The results of this comparison are enumerated in Tables I to XI.[3]

An inspection of these tables reveals the following facts:

1. In nine out of the eleven different experimental groups the long lived animals display evidence of superior growth velocity to the short lived animals at some time prior to the 30th week of age. Exceptions are afforded by the normal males and the lecithin-fed males.

2. At later ages there is a decided tendency for the curve of growth of the short lived animals to approach or cut across the curve which represents the growth of the long lived animals. This is due to the tendency of the short lived group to acquire a late accretion of tissue. This gain is, however, unstable and usually of comparatively brief duration, the curve dropping to the level of the long lived group or below it as the termination of life is approached.

3. In eight out of the eleven different experimental groups the long lived animals are less variable than the short lived animals. Exceptions are afforded by the normal females and both groups of tethelin-fed females.

4. Difference between the two groups is, however, most markedly displayed in their mode of reaction to external disturbing factors. All growth curves reveal minor fluctuations, accompanied by transient increases in variability, which are presumably attributable to unidentifiable fluctuations of the environment. The short lived animals are, however, much less stable than the long lived; their average weight is subject to more numerous and violent fluctuations and their variability increases abnormally towards the latter part of the curve. A disturbance of the environment may be reflected by the long lived group in enhanced

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[3] For details as to dosages of the dietary additions, number of animals employed, experimental procedure, *etc.* consult the previous articles of this series.

variability without concurrent alteration of weight, while the short lived animals simultaneously display relatively rapid gains or somewhat slower losses.[4]

Summarizing these results we may state therefore that as a general rule, when long and short lived animals which have been subjected to the same experimental treatment are compared, the long lived animals form a group which in early life grow more rapidly and at the same time are less variable than the short lived animals. In later life the short lived animals often grow much more rapidly than the long lived animals, but this accretion of tissue is relatively unstable.

So far we have restricted our comparison to the long and short lived animals respectively of the same experimental group. When we extend the comparison further and compare the long lived animals of one experimental group with those of another we find that there is no characteristic type to which the growth curve of long lived or short lived animals tends to conform. A glance at Figs. 1 and 2, in which all the various growth curves for males and females respectively are plotted to the same scale, shows that there is no tendency for the curves of the long lived animals to lie together or to be appreciably separated from the group of curves representing the growth of the short lived animals. On the contrary the curves are interwoven with one another. There is in fact much more tendency for a given curve to resemble its dietary similar than its longevity similar. Thus in both diagrams the curves for the long lived and short lived pituitary-fed animals tend to accompany one another, both as to form and position, and the same is true for the lecithin and tethelin curves. In other words the various dietaries employed in these experiments had a much more decided effect upon the time relations of growth than they had upon the longevity of the animals to which they were administered. This confirms the conclusion formulated in a previous article;[5] namely, that the substances administered, with the exception of tethelin, had no decided effect upon the life duration of the animals.

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[4] This is true long before the numbers of the short lived animals have been decreased by deaths.

[5] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 427.

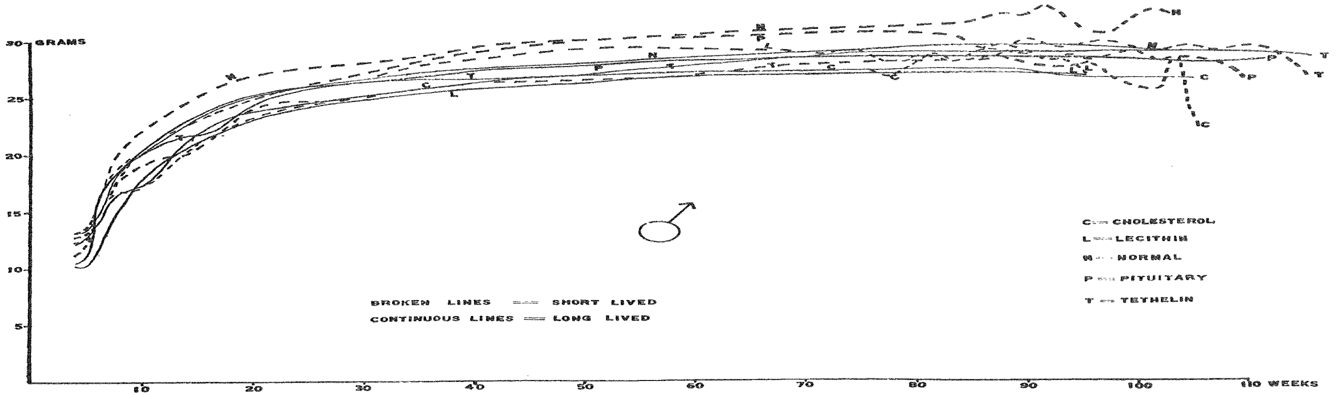

Fig. 1. Comparison of the growth curves of relatively long lived and relatively short lived males. There is no single type of curve pertaining to longevity.

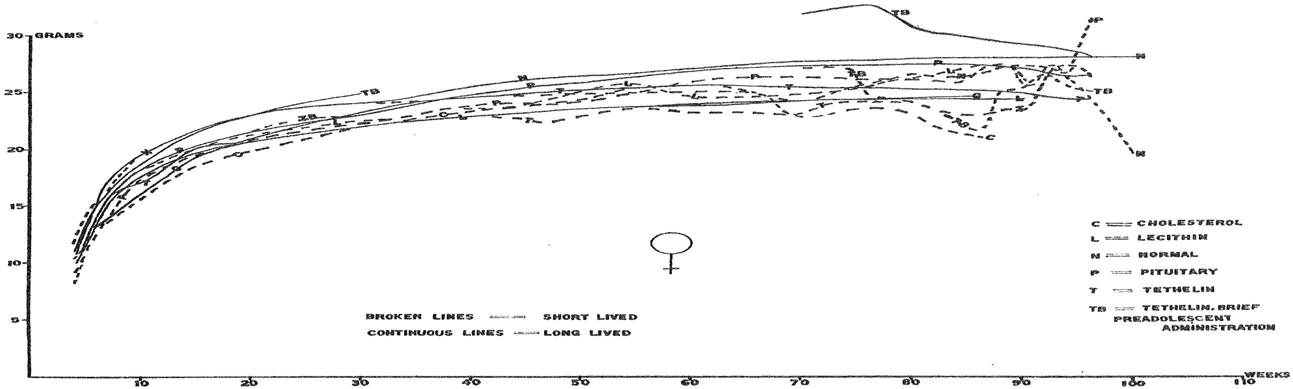

Fig. 2. Comparison of the growth curves of relatively long lived and relatively short lived females. There is no single type of curve pertaining to longevity. The supernormal growth of the long lived animals which received a brief preadolescent administration of tethelin is clearly displayed.

A correlation may nevertheless be traced between the time relations of growth and the longevity of the animals which display them. It is masked, but not concealed, by regulatory internal factors which tend to maintain the normal standard of longevity. Thus we have seen that in almost every group the long lived animals grow initially, or at an early stage, more rapidly than the short lived animals and that in comparatively late life the short lived animals display an unstable increase in weight. This late accretion of tissue would appear to be exceptionally unfavorable to longevity. Thus the longest lived animals in the series, those fed upon tethelin throughout the period of their lives (Table V) or intermittently (Table VI), do not display it at all, the curve of growth from the 40th to the 100th week being almost a straight line, and the average weight during this period increases very slightly.[6] The opposite extreme is afforded by those animals which have received tethelin for only a brief period antedating sexual maturity (4th to 12th weeks). As we have previously pointed out,[7] these animals display an extraordinary degree of late overgrowth, to which the curves marked TB in Fig. 2 bear striking witness.[8] These animals not only displayed no increased duration of life but were actually less viable than normal females, the short lived group especially being the least viable of all the experimental groups.

Increased viability is accompanied by a tendency for the short lived group to approach the long lived group in life duration, and conversely a brief average duration of life is accompanied by an enhanced difference between the viability of the two groups. This is shown by Table XII in which the different experimental groups are arranged in the descending order of their mean duration of life.

It is evident that the dispersion of deaths, as measured by the ratio of the difference between the life durations of the long and short lived animals to the mean life duration of the group, is least in the longest lived groups and progressively increases as the viability decreases. The increased scattering of deaths with decrease of viability is due to the fact that while the viability of the long lived groups is affected it is not so greatly affected by external or dietary circumstances as the viability of the short lived group. Thus in passing downwards among the males from an average life duration of 866 days to one of only 731 days, the life duration of the long lived group diminishes by 36 days, while that of the short lived group diminishes by 199 days. The same tendency is exhibited by the females (compare the tethelin continuous administration group with the tethelin brief administration) but not so conspicuously.

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[6] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 393.

[7] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 455.

[8] No measurements of the weights of these animals were made between the 30th and the 70th weeks.

Discussion and Interpretation of the Results.

In attempting the comparison of the growth of more viable as contrasted with that of less viable animals we are constrained to adopt some standard of viability which must of necessity be more or less arbitrary. The standard herein adopted is the average duration of life of any given experimental group and animals are grouped as long or short lived in comparison with this standard. It is the only practicable standard, and yet it is far from satisfactory, for a certain proportion of the short lived animals, that is, of the animals dying before the average life duration of the group, must necessarily die of causes which are essentially accidental although not clearly recognizable as such. Thus an animal which might otherwise have lived to the most advanced age afforded by the species develops a sarcoma or an infection and dies prematurely. We cannot definitely assert that in these cases, as in carcinoma, the lesion is consequent upon age or necessarily correlated with age. We can safely aver that senescence of the tissues must lay them open to disease, but we cannot conversely assert that disease proves senescence. But if these animals, dying of causes unconnected with the age of their tissues, were recognizable and excluded from the comparison, the average duration of life would thereby be advanced to a point which we have no means of ascertaining, and the more viable group would be correspondingly restricted in numbers.

If the true average duration of life of any group of animals, in so far as this is decided by tissue age alone, were ascertainable, therefore, the proportion of animals herein regarded as most viable would be reduced by exclusion of less viable individuals, while certain individuals would be excluded altogether from the less viable group or even included, despite their early death, among the members of the more viable group. All that the foregoing data can be expected to reveal, therefore, is a more or less pronounced *tendency* of the two groups to differ, a tendency which must be masked to a certain extent by the imperfect means of distinction employed.

This tendency is very plainly evinced and, as we have seen, it consists in initial relative supernormality of growth of the long lived group, accompanied by a subnormality of variability. The only exception to both these rules is afforded by the group of "normal males." The origin of this single exception among eleven different experimental groups may not improbably lie in the elements of uncertainty we have enumerated.

The association of superior growth with inferior variability is very remarkable, because it is precisely the opposite of what we find in comparisons of groups differentiated by any other criterion than that of longevity.[9] If different experimental groups or ages of animals or of man are compared, it is always found that the most rapidly growing and, age for age, the largest animals, are also the most variable.

The converse association, namely that of retarded growth with high variability, is characteristic of the effects of a highly unfavorable environment.[10] We may therefore infer that the long lived animals, displaying as they do the reverse tendency, are unusually immune to deleterious factors in their environment. They are a relatively stable group. Internal regulatory processes tend promptly and accurately to restore disturbed equilibria, and, as we have seen, this relative stability is also evidenced by the comparative rarity of irregular fluctuations of weight.

The relative internal stability of the long lived groups of animals is especially displayed by the events which occur at the period of life which corresponds with the average duration of life of the short lived groups. Factors which are at present unknown disturb at this point the stability of the animals, and this disturbance is reflected in increased variability of both the long lived and the short lived groups. But in the long lived groups this is accompanied by little or no disturbance of weight, while in the short lived group it results in loss, or in sharp gain followed by loss of weight.

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[9] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 363; *Am. J. Physiol.*, 1916, xli, 535. Thompson, D'A. W., On growth and form, New York, 1917.

[10] Robertson, T. B., *J. Biol. Chem.*, 1916, xxv, 635, 647; *Am. J. Physiol.*, 1916, xli, 547.

The long lived animals therefore represent a relatively stable group, highly resistant to external disturbing factors, displaying subnormal variability and a more or less marked, but not invariable, tendency to early overgrowth and relative paucity of tissue accretion in late life.

The short lived animals, on the contrary, are relatively unstable, sensitive to external disturbing factors, supernormally variable, and as a rule, but not invariably, display relatively deficient early growth and a tendency to rapid accretion of tissue in late life.

Before endeavoring to interpret these results we must revert to a peculiarity which distinguishes the action of agents known to affect the rate of growth of tissues and the longevity of animals to which they are administered. It has been shown in a number of previous communications that cholesterol[11] and tethelin[12] accelerate the growth and reproduction of unicellular organisms (*Paramecia*) and of epithelial tissues, especially carcinoma. Tethelin also accelerates the recovery of weight lost during inanition. Yet these agents which accelerate the development or multiplication of the tissue elements which we have enumerated nevertheless *retard* the growth in weight of animals to which they are administered by mouth.[13] We can only infer that there must be, in the totality of tissues which constitute the higher Metazoa, certain tissues, and those the relatively more bulky, which are retarded in their development, directly or indirectly, by these agents.

On considering the nature of the tissues which are positively known to be accelerated in their development by cholesterol and by tethelin the first point of resemblance which presents itself lies in the fact that they are all preeminently cellular or parenchymatous tissues or tissue elements. Now there exists in the body of all the higher Metazoa a considerable mass of tissue, usually the relatively more bulky, which is not primarily cellular or parenchymatous in character, but consists of fibrils or other specialized elements having, on the whole, a primarily structural rather than a primarily vital significance to the animal. They are produced from cell elements and ultimately derive from them the materials for their nutrition, maintenance, and repair; they are dependent tissues and may to a large extent be regarded as ultimately parasitic upon the parenchymatous elements.[14]

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[11] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1913, xvii, 344. Browder, A., *Univ. of California Pub. Physiol.*, 1915, v, 1. Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *J. Biol. Chem.*, 1915, xxi, 309.

[12] Robertson, T. B., *J. Am. Med. Assn.*, 1916, lxvi, 1009. Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1915, xxi, 280; 1916, xxiii, 631.

[13] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 397; 1919, xxxvii, 393. Robertson, T. B., and Delprat, M., *J. Biol. Chem.*, 1917, xxxi, 567.

These primarily structural or sclerous elements of the body are constituted for the most part of substances which are characteristic of the particular type of tissue which contains them, substances which contribute to its peculiar physical properties, its rigidity, elasticity, tensile strength, or other essential quality. This is especially true of the protein constituents of these tissues and it is precisely from tissues of this character that we obtain aberrant or "incomplete" proteins, deficient in this or that amino-acid and characterized by their abnormal content of this or that other amino-acid. To produce such protein of abnormal composition requires the destruction and resorting of the components of several or perhaps many molecules of the more "typical" proteins which occur in parenchymatous tissue. The connective tissues are therefore relatively expensive to support and dependent as they are, directly or indirectly, upon parenchymatous tissues for their maintenance they must cast collectively a heavy load upon the metabolism and maintenance of the most vital tissues in the body.

In any system of parallel chemical reactions which consume the same substrate and are proceeding simultaneously in a mixture, the extent to which each reaction will occur is proportionate to its velocity.[15] The most rapid reaction will predominate over the rest and appropriate the greater share of the substrate. In the tissues of the Metazoa, the substrates are the foodstuffs which are present in the circulating fluids which bathe the tissue elements and the reactions of synthesis which underlie growth, repair, and maintenance share among them in a definite proportion the various nutritive materials which are available. If, therefore, any circumstance, such as the introduction of a catalyzer, specifically accelerates the syntheses occurring in one type of tissue to the exclusion of others, this type of tissue will acquire an advantage in the appropriation of nutriment and the other tissues will in like proportion be retarded in their development, maintenance, or repair.

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[14] Adami, J. G., Principles of pathology, Philadelphia and New York, 1908, i, 125.

[15] Mellor, J. W., Chemical statics and dynamics, London, New York, and Bombay, 1904, 70.

We have seen that the cellular tissues are definitely accelerated in their growth and repair by cholesterol and tethelin, while the growth of animals as a whole is retarded by these agents. It seems necessary to infer that certain tissues other than the distinctively cellular elements are retarded in their development by cholesterol and tethelin. The only considerable group to which we can attribute this retardation of growth in weight is the group of sclerous or dependent tissues.

An increase in connective tissue elements is commented upon by all observers[16] as the most distinctive characteristic of old age, and when we reflect that these tissues are ultimately dependent upon parenchyma for their support and that they are exceptionally expensive to maintain we can readily comprehend how an ever increasing accumulation of tissues of this character may place an excessive and finally unbearable load upon the metabolism of the cellular tissues. On the other hand a very high proportion of cellular elements is characteristic of embryonic tissue.

If this view is correct, then the problem of the prolongation of life is essentially that of deferring the accretion of connective tissues which occurs in the later life of all the higher Metazoa. If we have correctly interpreted the influence of tethelin upon the growth of animals as consisting in acceleration of the anabolism of the cellular elements to the detriment of the connective tissue elements, then the decided prolongation of life which administration of this agent brings about[5] is readily comprehended. Cholesterol, which in like manner but in greater dosage accelerates the growth of cellular tissues, does not bring about a like prolongation of life, but for this there is a sufficient reason; namely, that deposits of this insoluble material are formed in various organs, to their detriment, when it is administered in excess.[17]

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[16] Saundby, R., Old age; its care and treatment in health and disease, London, 1913, Chapter 2. Metchnikoff, E., The nature of man, New York and London, 1913; The prolongation of life, New York and London, 1910.

If, however, the stimulus supplied by tethelin to the anabolism of cellular elements is removed, at any rate if it is removed before sexual maturity is attained, the normal proportionality between cellular and sclerous elements tends rapidly to be restored; the overgrowth which has actually occurred but has been masked by the retarded development of sclerous tissues is now rendered manifest, and gigantic animals may be produced. In illustration Fig. 3 shows a normal female of average normal weight at 500 days in comparison with a female of the average weight of twenty-four animals which had received 4 mg. of tethelin daily from the 4th until the 12th week after birth, the administration being thereafter discontinued. These animals exhibited an abbreviated rather than a prolonged duration of life.

Reverting to the experimental data presented in this paper it will be seen that while the long lived animals in any experimental group usually exhibited initial overgrowth in comparison with the short lived animals they displayed much less tendency to form late accretions of tissue. Bearing in mind the fact that the late growth of animals consists predominantly in connective tissue formation, these facts would appear to harmonize with the view that the potential longevity of any given individual is determined by the relative velocities of anabolism in the cellular and sclerous tissues. A high rate of cellular anabolism, while encouraging the growth of distinctively cellular tissues, defers the connective tissue accretion which is characteristic of senescence. A low rate of cellular anabolism renders available a higher proportion of nutrients for the distinctively structural tissues and necessitates premature senescence.

Since longevity is determined, not by the absolute mass of tissue, but by the relative proportion of parenchymatous to sclerous tissues, there is no single magnitude or contour of growth curve which is characteristic of long lived animals, and for the same reason the longevity of different species of animals bears no relation to their mass or bulk. On the other hand the longevity of different species has been shown to stand in definite relation to the proportionality between different types of tissue; *e.g.*, of brain weight to total body weight.[18]

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[17] Chalatow, S. S., *Virchows Arch. path. Anat.*, 1912, ccvii, 452; *Beitr. path. Anat. u. Path.*, 1914, lvii, 85. Anitschkow, N., *Beitr. path. Anat. u. Path.*, 1913, lvi, 379; 1914, lvii, 201. Weltmann, O., and Biach, P., *Z. exp. Path. u. Therap.*, 1913, xiv, 367.

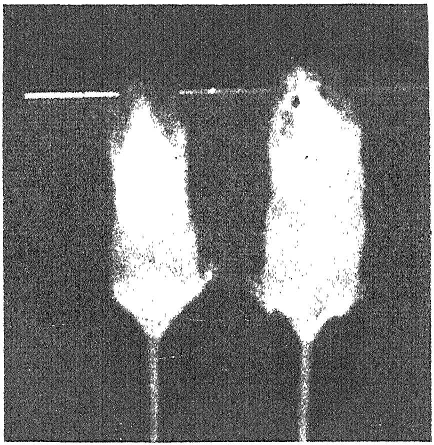

FIG. 3. Photograph of two female mice, each 500 days old. The smaller animal (25 gm.) is the average weight of a normal female of this age. The larger (31 gm.) has received a brief preadolescent administration of tethelin (8th to 12th weeks) which was thereafter discontinued. Its weight is the average weight of twenty-four animals similarly treated. The discontinuation of the tethelin before sexual maturity has permitted the actual supernormality of growth induced by this agent to express itself by the reestablishment of the normal proportion of sclerous to parenchymatous tissues which the continuous administration of tethelin prevents.

SUMMARY.

1. In any given group of animals those which live longest represent a relatively stable subgroup, highly resistant to external disturbing factors, displaying subnormal variability and a more or less marked, but not invariable, tendency to early overgrowth and relative paucity of tissue accretion in late life.

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[18] Friedenthal, H., *Centr. Physiol.*, 1910–11, xxiv, 321.

2. The short lived animals, on the contrary, are relatively unstable, sensitive to external disturbing factors, supernormally variable and, as a rule but not invariably, display relatively deficient early growth and a tendency to rapid but unstable accretions of tissue in late life.

3. The hypothesis is presented that the potential longevity of any given individual is determined by the relative velocities of anabolism in the cellular and sclerous tissues respectively.

4. In terms of this hypothesis it is suggested that the prolongation of life which is brought about by continuous administration of tethelin to mice is attributable to an increased velocity of anabolism of distinctively cellular elements to the relative disadvantage, in the competition for nutrients, of the connective tissue elements.

5. If the administration of tethelin is discontinued prior to sexual maturity the normal proportion of cellular to connective tissues is rapidly restored and exceptionally large animals of normal or abbreviated duration of life are the result.

TABLE I.

Normal Males.
(Average duration of life 767 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	10.50	12.38		24.4
5	11.05	12.45	20.1	24.6
6	15.39	15.58	22.7	22.0
7	17.25	18.57	17.6	16.9
8	18.28	20.02	14.9	15.9
9	19.38	21.40	18.4	16.5
10	20.03	21.90	17.6	16.7
11	20.75	22.46	14.4	13.3
12	21.84	23.15	14.5	13.7
13	22.34	24.05	15.8	14.2
14	22.81	24.88	14.6	14.1
15	23.34	25.03	12.8	11.9
16	23.78	25.53	13.2	12.7
17	24.00	26.18	13.2	12.4
18	24.84	26.23	13.8	12.1
19	24.69	26.76	13.0	12.1
20	25.28	26.79	12.4	10.8
21	25.50	26.95	12.2	11.2
22	25.31	26.68	10.6	9.0
23	25.47	27.03	11.6	9.8
24	26.09	27.47	11.6	10.1
25	26.03	27.89	12.1	11.0
26	26.06	27.76	10.7	10.8
27	25.33	27.62	12.6	11.3
28	26.27	28.06	11.0	10.9
29	25.90	28.19	10.3	10.9
30	26.50	27.91	9.8	9.5
32	26.97	28.25	9.8	9.6
34	27.00	28.47	9.9	10.5
36	27.31	28.68	10.2	10.1
38	27.41	29.11	10.3	10.5
40	27.28	29.39	9.6	10.4
42	27.34	29.36	10.7	11.2
44	27.50	29.89	12.0	12.0
46	27.75	29.46	11.7	11.7
48	27.44	29.11	10.5	10.8
50	27.97	29.93	11.3	11.1
52	27.66	29.86	10.1	10.7
54	27.47	30.04	11.6	12.0

TABLE I—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	28.28	30.50	11.8	12.0
58	28.34	29.96	11.3	10.9
60	28.13	30.18	11.4	11.8
62	28.47	30.50	10.8	11.6
64	28.53	30.68	10.2	11.4
66	28.56	31.00	10.2	11.4
68	28.72	31.04	10.0	11.1
70	28.81	30.71	9.9	10.9
72	28.59	31.00	11.5	12.2
74	28.84	30.92	9.8	10.9
76	29.28	30.85	9.4	10.0
78	29.13	31.42	9.5	11.5
80	29.25	30.81	10.4	11.1
82	29.59	31.45	9.7	11.3
84	29.28	30.85	10.7	12.3
86	29.44	31.28	10.4	13.0
88	29.19	32.19	11.1	13.0
90	29.25	31.88	10.8	11.3
92	29.28	32.81	9.0	12.0
94	29.28	31.81	9.6	11.1
96	29.25	30.64	9.6	10.1
98	29.00	30.33	9.6	10.0
100	28.91	31.60	8.6	9.6
102	28.75	32.38	10.3	10.7
104	29.19	31.83	9.7	10.4

Summary of Table I.—The long lived animals form a group which are initially lighter than the short lived animals and do not grow more rapidly so that the relative subnormality is maintained throughout life. They are less variable than the short lived animals initially and after the 30th week, but between the 9th and 30th weeks they are more variable than the short lived group.

TABLE II.

Normal Females.

(Average duration of life 719 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	11.21	10.39	23.9	23.4
5	13.64	11.81	21.2	19.4
6	15.50	14.12	17.7	18.9
7	17.17	16.39	15.8	15.0
8	18.14	17.83	14.4	13.9
9	18.64	18.92	14.3	13.4
10	19.31	19.44	15.0	14.3
11	20.03	20.05	13.4	12.6
12	20.31	20.31	13.6	12.6
13	20.89	21.19	13.8	13.0
14	21.28	21.14	15.0	13.2
15	21.78	21.78	14.2	13.7
16	22.00	22.28	13.2	12.6
17	22.28	22.31	13.2	11.9
18	22.47	21.97	13.2	11.9
19	22.67	22.53	12.9	12.0
20	22.50	22.70	12.8	11.2
21	23.19	22.92	13.6	12.3
22	23.28	23.36	14.2	12.5
23	23.59	23.44	14.1	12.0
24	23.67	23.69	13.0	11.3
25	23.82	23.75	13.0	11.4
26	24.08	24.00	12.0	11.6
27	23.68	24.31	13.8	11.8
28	23.64	23.53	11.9	10.3
29	24.00	23.69	13.4	12.2
30	23.92	23.92	14.0	12.4
32	24.25	24.11	13.6	11.5
34	24.19	24.17	13.8	12.2
36	24.75	24.56	13.0	11.2
38	25.17	24.41	14.1	12.3
40	25.50	24.53	14.1	11.9
42	25.58	24.53	15.6	13.1
44	26.06	24.91	17.7	15.3
46	26.31	24.93	16.6	14.6
48	26.08	24.64	16.4	14.6
50	26.03	24.82	15.8	14.0
52	26.22	25.00	16.9	15.3
54	26.47	24.64	17.4	15.8

TABLE II—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	26.67	24.91	17.8	16.5
58	26.94	25.14	17.9	16.7
60	27.00	24.68	18.6	17.8
62	27.28	24.95	19.2	17.7
64	27.58	25.09	18.8	17.8
66	27.50	24.55	19.8	19.2
68	27.58	24.70	18.5	18.3
70	27.22	23.94	18.9	18.7
72	27.36	24.75	18.4	17.7
74	27.42	25.07	17.8	16.9
76	27.83	25.71	18.7	17.7
78	27.86	25.70	17.6	17.0
80	27.92	26.38	18.4	17.4
82	27.69	26.00	17.9	17.0
84	27.89	27.13	17.2	16.4
86	28.00	27.38	18.9	17.6
88	27.92	27.13	19.8	18.7
90	27.97	27.13	19.2	16.8
92	28.08	27.33	19.3	18.4
94	27.81	25.00	18.5	18.0
96	28.31	25.50	18.4	17.8
98	28.22	22.00	20.6	20.9
100	27.86	19.50	21.4	22.2

Summary of Table II.—The long lived animals are initially heavier than the short lived animals. From the 9th till the 36th weeks the weights of the two groups are approximately equal. Thereafter the long lived animals grow more rapidly than the short lived animals so that by the 70th week they exceed the short lived animals by over 3 gm. The variability of the long lived group slightly exceeds that of the short lived group until the 66th week, after which the variabilities are approximately equal. Death in the short lived group is preceded (70th week onward) by a sharp rise of weight.

TABLE III.

Pituitary-Fed Males.
(Average duration of life 792 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	12.13	13.21	13.7	22.7
5	12.72	13.45	19.0	24.1
6	15.59	16.08	18.8	22.1
7	17.28	17.45	15.8	17.7
8	18.72	18.50	17.4	17.3
9	19.22	19.80	15.4	14.8
10	20.03	20.03	15.2	15.4
11	20.66	19.53	15.3	14.9
12	21.84	21.75	9.4	11.4
13	22.06	22.60	8.4	10.7
14	22.91	23.08	8.0	10.0
15	22.88	23.20	7.4	9.8
16	23.66	23.68	7.6	9.3
17	24.10	24.12	8.2	9.9
18	24.25	24.10	8.1	9.7
19	24.44	24.68	9.0	10.2
20	24.44	24.78	8.5	9.8
21	25.06	24.93	8.4	9.6
22	25.47	25.25	9.8	11.5
23	25.91	25.80	7.7	8.7
24	25.97	26.38	7.7	10.5
25	25.81	26.00	9.1	9.8
26	25.72	26.43	7.6	9.2
27	26.00	26.20	7.9	8.8
28	26.17	26.39	7.3	8.7
29	26.06	26.37	8.1	8.8
30	26.21	26.68	9.2	8.4
32	26.38	26.93	8.3	9.0
34	26.81	27.83	8.7	9.7
36	26.78	27.88	7.6	9.3
38	27.00	28.72	8.4	9.3
40	27.34	28.72	7.7	9.2
42	27.47	28.67	8.6	10.6
44	27.53	29.17	8.1	10.0
46	27.53	29.00	8.3	10.1
48	27.13	29.11	8.7	11.1
50	27.53	29.54	8.8	11.2
52	27.66	29.64	8.1	11.2
54	28.09	29.86	9.6	11.4

TABLE III—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	28.06	29.86	8.4	11.1
58	28.50	30.32	8.7	11.0
60	28.44	30.54	8.4	11.0
62	28.19	30.18	7.8	10.6
64	28.19	30.57	7.8	11.3
66	28.75	31.00	7.6	11.4
68	28.88	30.50	8.0	11.4
70	28.41	30.46	8.0	11.9
72	28.28	30.96	8.3	11.0
74	28.72	30.88	9.0	11.5
76	28.25	30.50	8.0	11.2
78	28.63	30.55	9.4	10.2
80	28.22	30.18	7.7	9.8
82	28.22	30.23	7.6	10.1
84	28.78	30.14	8.0	10.2
86	28.00	29.15	7.5	8.2
88	28.50	29.39	8.1	9.0
90	28.81	29.06	8.3	8.6
92	28.03	28.06	7.3	6.5
94	28.44	28.44	7.8	7.9
96	28.38	28.13	8.0	7.8
98	28.06	28.43	8.4	8.4
100	27.94	28.93	7.5	8.1
102	28.38	28.10	7.5	9.2
104	27.78	28.30	7.5	7.5
106	27.97	28.10	6.6	8.1
108	27.97	27.75	6.8	6.7
110	28.00	26.50	7.1	7.6
112	28.04		8.2	6.3

Summary of Table III.—The long lived animals are initially lighter than the short lived animals, but rapidly gain on them, so that by the 10th week the two groups are equal in weight. The long lived never surpass the short lived animals, however, and by the 24th week begin to drop behind and by the 40th week and thereafter are markedly lighter than the short lived group. The long lived animals are consistently much less variable than the short lived animals.

TABLE IV.

Pituitary-Fed Females.
(Average duration of life 704 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	9.84	9.04	18.5	20.5
5	12.28	11.07	16.8	19.6
6	14.35	13.27	13.3	16.1
7	15.40	13.73	13.8	15.8
8	16.79	15.47	12.4	15.1
9	17.64	16.63	11.0	13.1
10	18.40	17.27	11.0	12.1
11	18.52	17.83	8.7	10.5
12	19.19	18.43	7.7	9.3
13	19.57	18.70	7.8	10.3
14	19.95	19.03	7.3	9.4
15	19.93	19.43	9.4	10.4
16	20.17	19.53	7.7	9.3
17	20.83	20.00	7.6	9.6
18	21.07	20.23	7.7	9.7
19	21.45	20.77	7.9	9.7
20	21.52	20.77	7.5	9.1
21	21.58	21.03	8.6	9.6
22	21.90	21.33	8.7	10.2
23	21.85	21.43	8.0	9.9
24	22.40	21.60	8.6	10.2
25	22.30	21.70	8.3	10.4
26	22.43	21.63	9.1	10.5
27	22.58	21.90	8.8	10.3
28	22.98	21.93	8.0	10.0
29	22.75	21.90	8.4	10.3
30	23.05	22.71	8.3	9.6
32	23.50	22.47	8.8	10.8
34	23.81	22.87	9.1	10.8
36	24.14	22.90	9.7	10.9
38	24.29	23.63	11.2	12.2
40	24.74	23.86	11.4	11.8
42	24.88	23.83	11.6	12.4
44	25.12	23.81	11.8	13.1
46	25.19	23.85	11.0	13.2
48	25.26	23.88	12.3	13.7
50	25.81	24.69	12.6	13.3
52	26.40	24.57	15.2	15.4
54	26.05	25.42	14.8	15.2

TABLE IV—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	26.60	25.13	14.9	15.0
58	26.57	25.71	16.1	15.9
60	26.90	26.10	13.2	14.5
62	27.12	25.80	15.1	16.0
64	26.81	25.75	14.8	16.3
66	27.19	26.61	16.1	16.8
68	27.33	27.19	16.4	16.9
70	27.24	26.01	16.2	17.0
72	27.02	26.75	15.6	16.6
74	27.26	26.56	16.6	16.8
76	27.17	25.83	16.1	15.7
78	26.93	26.00	13.9	13.9
80	27.21	25.58	16.2	16.2
82	27.48	26.00	18.0	17.6
84	27.43	25.60	17.9	17.6
86	27.33	27.38	18.8	17.6
88	27.26	27.50	15.2	14.4
90	27.07	25.50	16.0	15.6
92	26.48	27.25	12.0	11.6
94	26.53	26.50	12.2	11.8
96	26.53	31.50	14.2	13.2

Summary of Table IV.—The long lived animals form a relatively invariable group, in which preadolescent growth is unusually rapid. The lead thus gained is maintained throughout life. The death of the short lived animals is preceded by a marked gain in weight accompanied by an increase in variability. The same increase in variability is noted at the same time in the long lived group, but the increase in weight is relatively less, and less subject to fluctuation than in the short lived group.

TABLE V

Tethelin-Fed Males; Continuous Administration.
(Average duration of life 866 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	13.36	12.80		
5	13.14	12.94	19.9	21.8
6	14.46	14.95	19.2	18.2
7	16.69	16.36	14.1	14.6
8	18.62	17.73	9.0	12.0
9	19.23	18.68	6.8	10.2
10	19.92	19.00	6.8	10.8
11	20.27	19.50	6.7	10.9
12	21.08	19.95	7.6	10.6
13	21.50	20.14	5.8	10.5
14	21.62	20.00	7.5	11.2
15	21.19	21.23	8.9	9.1
16	22.19	21.23	7.3	8.4
17	22.50	22.82	8.0	8.0
18	23.12	23.32	6.9	7.3
19	23.92	23.64	7.0	7.2
20	24.65	24.00	5.4	6.3
21	24.65	24.36	5.0	6.2
22	25.00	24.70	6.4	7.6
23	24.77	24.40	5.4	6.4
24	25.27	24.45	7.2	7.7
25	25.73	24.80	6.2	6.7
26	25.69	24.80	6.8	8.1
27	26.08	24.95	7.0	8.0
28	25.85	24.95	6.7	7.4
29	26.15	24.95	6.4	7.9
30	26.00	25.28	6.8	7.2
32	26.46	25.40	5.7	7.1
34	26.69	25.60	6.9	8.8
36	26.31	25.65	6.2	6.9
38	26.27	26.20	6.7	7.4
40	26.54	26.30	5.1	5.4
42	26.65	26.40	5.8	5.8
44	26.81	26.10	6.3	6.9
46	27.04	26.35	7.1	7.0
48	26.88	26.65	6.4	6.9
50	26.85	26.10	7.7	7.8
52	27.35	26.50	7.5	7.2
54	27.81	27.40	6.3	7.0

TABLE V—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	28.04	27.30	7.8	7.7
58	27.77	26.75	6.4	6.7
60	27.23	26.55	7.4	7.3
62	27.81	27.00	6.3	7.0
64	27.73	27.45	7.0	7.2
66	27.62	26.70	6.9	7.9
68	28.31	27.75	6.5	7.9
70	28.42	27.70	6.9	7.8
72	28.42	27.60	7.3	7.8
74	28.46	27.30	7.6	8.4
76	28.88	28.05	6.4	7.7
78	28.54	27.70	7.2	8.2
80	28.96	28.11	7.3	9.2
82	28.81	28.00	5.3	7.7
84	28.19	28.00	6.4	7.9
86	28.62	28.00	7.2	8.8
88	28.62	27.56	6.7	8.4
90	29.04	29.92	6.8	7.9
92	28.81	28.84	7.6	8.4
94	28.58	29.25	6.9	8.2
96	28.58	29.60	5.9	8.7
98	28.62	29.60	9.3	10.1
100	28.65	29.10	6.6	8.2
102	28.50	28.80	6.1	8.2
104	28.85	29.40	6.0	8.5
106	28.69	29.00	7.9	9.2
108	28.81	29.00	6.7	7.9
110	28.58	29.20	6.8	7.9
112	28.77	28.70	6.8	7.8
114	28.85	28.40	7.0	7.3
116	28.31	26.50	7.0	6.7

Summary of Table V.—The long lived animals form a relatively invariable group in which preadolescent growth is unusually rapid. The lead thus established is maintained in diminishing degree throughout life. Death of the short lived group is preceded by a marked gain in weight, both absolutely and relatively to the long lived group.

TABLE VI.

Tethelin-Fed Females; Discontinuous Administration 4th to 8th, 21st to 25th, and 42nd to 46th Weeks.

(Average duration of life 800 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	10.50	11.60	28.8	27.6
5	13.13	13.75	22.6	19.6
6	14.56	15.31	22.0	19.4
7	15.88	15.69	17.9	16.1
8	16.06	16.31	15.8	12.6
9	16.56	16.69	15.2	12.4
10	17.00	17.06	15.0	13.2
11	17.56	17.50	18.1	15.7
12	17.88	17.44	19.2	16.6
13	18.94	19.00	16.3	14.1
14	19.75	19.69	13.0	11.9
15	20.56	20.50	13.4	11.4
16	20.31	20.06	12.8	11.2
17	21.00	20.19	12.4	11.5
18	20.31	20.31	11.6	10.7
19	20.63	20.56	14.1	11.9
20	20.88	20.31	14.0	12.1
21	21.31	20.88	11.9	11.2
22	21.50	20.75	12.0	11.1
23	21.88	21.06	13.6	12.2
24	22.13	21.44	14.7	12.7
25	22.38	21.13	13.6	12.8
26	22.56	21.44	14.8	13.5
27	22.50	21.19	14.4	13.6
28	22.81	21.63	15.8	14.2
29	22.57	22.14	16.0	14.1
30	23.25	21.50	17.4	15.4
32	23.63	21.56	16.9	15.4
34	23.94	22.19	17.6	15.7
36	24.06	21.69	16.4	15.7
38	24.25	22.63	16.9	15.6
40	24.19	22.88	18.8	16.9
42	24.44	22.63	19.0	17.9
44	25.19	22.69	19.2	18.8
46	25.19	22.00	17.6	17.8
48	24.56	22.44	16.4	17.1
50	25.31	22.88	16.5	17.6
52	25.56	23.25	16.5	16.6

TABLE VI—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
54	25.38	23.44	18.5	17.2
56	25.38	23.75	16.6	17.1
58	25.50	23.25	16.7	16.7
60	25.56	23.06	16.7	16.8
62	25.38	23.19	15.6	16.8
64	25.50	23.19	15.2	16.7
66	25.13	23.19	14.5	16.1
68	25.19	22.94	13.8	14.7
70	25.00	23.13	14.0	15.4
72	25.88	24.14	15.4	16.3
74	25.31	24.00	14.3	15.5
76	25.63	24.07	14.5	15.7
78	25.25	24.14	14.1	15.5
80	25.38	24.07	15.3	16.4
82	24.50	23.29	14.4	16.0
84	25.13	23.00	14.4	16.6
86	25.94	22.80	13.9	16.1
88	25.63	23.60	15.5	17.9
90	25.25	23.40	12.6	16.8
92	24.25	26.67	14.4	15.1
94	24.69	27.33	12.0	12.5
96	24.50	26.25	13.6	12.6

Summary of Table VI.—The long lived animals are at first lighter and more variable than the short lived animals. From 23 weeks onwards, however, the long lived group not only catch up to but notably surpass the short lived group. At this stage they therefore grow more rapidly than the short lived animals. They remain more variable until the 44th week. Death, in the short lived group, is preceded by a small increase of weight at 72 weeks and at 92 weeks by a large and sudden increase which is in part but not wholly due to the death of the lighter individuals.

TABLE VII.

Tethelin-Fed Females; Brief Preadolescent Administration 4th to 12th Weeks Only.

(Average duration of life 695 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	10.90	10.31	7.9	9.0
5	13.35	11.77	18.8	20.0
6	14.27	13.50	14.8	16.2
7	16.12	14.86	13.8	15.9
8	17.00	16.46	13.0	15.0
9	17.81	16.55	13.6	14.8
10	18.27	16.77	12.5	14.2
11	19.08	17.50	11.7	13.4
12	20.00	18.75	13.0	13.6
13	20.62	19.00	10.5	11.8
14	21.15	19.50	13.2	13.3
15	21.15	19.95	12.6	12.6
16	21.77	20.10	11.8	11.7
17	21.92	20.85	11.4	11.4
18	22.65	21.10	11.4	12.0
19	23.00	21.80	11.4	12.1
20	22.77	21.45	10.8	11.9
21	23.23	22.00	12.2	12.7
22	23.35	21.80	11.4	12.4
23	23.38	22.05	10.9	12.1
24	23.65	22.65	12.0	12.3
25	23.88	22.65	12.9	13.0
26	24.35	22.75	11.8	12.6
27	24.12	22.75	14.0	14.3
28	24.35	22.60	13.6	15.1
29	24.04	22.85	12.4	13.3
30	24.88	22.85	13.4	14.7
No measurements were made between the 30th and 70th weeks.				
70	31.81	27.00	19.4	23.2
72	32.23	26.33	19.1	23.5
74	32.46	27.00	18.9	19.5
76	32.54	24.50	18.9	20.2
78	31.73	24.75	15.6	18.2
80	30.54	24.00	17.8	18.2
82	30.62	23.17	17.4	19.2
84	30.08	22.67	17.4	19.6

TABLE VII—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
86	29.85	21.50	17.1	20.7
88	29.38	25.00	16.8	16.9
90	29.38	25.25	17.4	17.4
92	28.92	26.50	17.8	17.4
94	28.81	25.50	17.8	17.6
96	28.15	25.00	16.4	15.8

Summary of Table VII.—The long lived animals form a group in which preadolescent growth is comparatively rapid and variability low. This superiority of growth is maintained and markedly enhanced in later ages, and the long lived animals remain the less variable until the average duration of life of the short lived group. The death of the short lived group may be preceded by a relatively sudden increase of weight (see the 70th to 74th weeks), but the lack of weighings between the 30th and 70th weeks renders it difficult to be sure of this. A sharp decrease of weight follows and slightly, but only slightly, precedes a similar loss of weight in the long lived group.

TABLE VIII.

Cholesterol-Fed Males.

(Average duration of life 764 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
5	12.83	12.85	19.2	18.1
6	13.98	14.19	17.0	19.3
7	15.75	16.00	14.2	16.2
8	16.80	17.00	13.7	15.5
9	16.85	16.71	16.4	17.9
10	17.38	17.46	17.6	18.4
11	17.88	17.82	16.9	18.2
12	19.23	18.57	14.2	16.1
13	20.70	20.11	11.5	13.5
14	21.88	20.64	10.4	12.4
15	22.33	21.25	11.7	13.3
16	22.33	21.61	10.3	12.9
17	22.34	22.00	9.4	11.0
18	23.25	22.89	10.5	11.5
19	23.05	21.81	7.6	9.9
20	23.90	22.61	8.2	11.0
21	23.67	23.17	7.0	9.1
22	24.58	23.64	9.6	10.5
23	24.03	24.00	7.3	9.4
24	24.56	24.23	9.7	10.3
25	24.24	24.25	6.9	8.9
26	24.64	24.23	9.2	10.5
27	24.12	24.21	6.8	9.2
28	25.22	24.64	9.2	10.4
29	24.35	24.92	7.2	9.1
30	24.82	24.67	10.5	11.2
32	25.38	25.29	10.1	11.1
34	25.53	25.75	10.5	11.3
36	25.88	25.36	11.0	11.8
38	26.08	26.35	9.8	9.9
40	26.07	26.04	12.7	11.8
42	26.38	25.88	11.0	11.5
44	26.03	26.67	10.1	10.4
46	27.25	26.58	9.8	11.7
48	26.90	26.73	8.6	9.4
50	27.05	26.46	8.5	9.3
52	26.85	26.67	9.4	10.5
54	27.63	27.29	8.6	9.9
56	26.93	27.08	9.1	9.8

TABLE VIII—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
58	26.75	26.83	9.8	11.3
60	26.93	26.79	9.5	10.8
62	27.05	27.08	9.2	10.1
64	27.08	26.83	8.5	10.2
66	27.03	26.83	9.5	10.8
68	26.93	27.29	8.6	10.9
70	27.23	27.23	8.3	10.2
72	27.15	27.36	9.5	10.9
74	27.33	27.27	8.6	11.1
76	27.33	28.17	9.5	10.8
78	27.03	26.72	10.2	11.1
80	27.25	27.78	9.2	10.3
82	27.35	28.63	9.6	10.9
84	26.83	28.00	10.5	11.8
86	26.98	28.14	10.8	12.4
88	27.43	28.36	10.4	11.3
90	27.15	27.79	11.0	12.3
92	26.83	27.50	10.2	12.1
94	26.60	27.42	12.0	13.7
96	26.53	27.75	13.2	13.0
98	26.43	26.60	12.0	13.4
100	26.28	25.83	13.4	14.4
102	26.53	25.33	12.2	13.9
104	26.25	28.25	12.4	13.6
106	26.65	22.00	12.0	12.4

Summary of Table VIII.—The long lived animals are initially smaller but soon thereafter grow more rapidly than the short lived animals and are less variable. The superiority of the long lived group is transient and disappears at about 25 weeks. Death of the short lived group is preceded by a relatively sudden increase in weight (80th week), the long lived animals displaying a simultaneous increase of variability but no increase in weight.

TABLE IX.

Cholesterol-Fed Females.

(Average duration of life 658 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
5	14.08	12.44	18.6	21.5
6	13.22	13.00	14.4	15.6
7	14.13	13.71	15.6	16.5
8	15.09	14.74	13.9	15.4
9	15.25	14.97	17.4	17.8
10	15.75	15.37	13.8	15.2
11	16.56	15.63	15.6	18.1
12	17.53	16.74	13.6	16.1
13	18.22	17.32	14.9	16.9
14	18.94	18.05	14.1	14.7
15	18.94	18.63	13.0	13.7
16	19.59	18.89	12.2	13.3
17	19.88	18.76	12.5	12.8
18	20.19	19.19	12.4	12.8
19	19.94	19.35	11.8	12.3
20	20.44	19.83	12.4	12.1
21	20.84	19.68	12.8	12.9
22	20.59	20.14	12.2	12.4
23	20.88	20.12	12.4	12.4
24	21.00	20.64	15.2	14.3
25	21.13	20.76	14.6	14.5
26	21.33	21.17	14.4	14.7
27	21.53	21.12	13.3	14.8
28	21.60	21.53	14.9	15.7
29	21.69	21.56	12.0	15.2
30	22.00	22.13	15.6	16.6
32	21.94	22.21	13.6	17.3
34	22.16	22.41	13.5	18.4
36	22.22	22.59	13.6	19.9
38	22.75	23.03	13.8	20.4
40	22.69	23.06	13.6	21.0
42	22.66	23.17	14.3	22.8
44	22.81	23.86	13.4	23.1
46	23.75	24.61	13.6	23.0
48	23.53	24.27	13.3	23.4
50	23.63	24.17	12.2	23.0
52	23.44	24.95	12.9	22.4
54	23.97	25.54	13.0	21.9
56	23.69	25.18	13.9	23.1

TABLE IX—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
58	23.38	25.35	13.7	23.0
60	23.84	25.15	14.0	21.1
62	24.16	25.56	12.6	22.3
64	23.94	25.17	13.4	21.7
66	23.97	24.83	14.1	21.6
68	24.25	23.14	14.8	15.5
70	24.34	22.79	15.0	15.5
72	24.66	23.29	16.3	16.9
74	24.38	23.29	14.3	15.8
76	24.19	23.29	16.1	16.8
78	24.13	23.57	14.7	16.0
80	24.50	22.83	16.9	17.9
82	24.75	21.75	16.4	18.5
84	24.69	21.33	16.8	19.1
86	24.44	21.00	16.4	19.3

Summary of Table IX.—Prior to the 30th week the long lived animals grow more rapidly than the short lived animals and they are less variable. The unusually early death of the short lived animals in this group is preceded by a remarkable gain of weight which enables them at a comparatively early stage to catch up to and surpass the long lived animals. This increase in weight is accompanied by an extraordinary concurrent increase of variability which is not perceived in the long lived group until they have attained over double the age (60 weeks). This confirms the view expressed in previous publications that the high variability of cholesterol-fed animals is due to deleterious effects of the cholesterol, since those animals which suffered from these effects most markedly also displayed the enhanced variability at an early age.

TABLE X.

Lecithin-Fed Males.

(Average duration of life 731 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	10.36	11.18	9.6	23.1
5	10.22	12.58	20.2	23.2
6	11.53	12.63	19.1	21.7
7	13.72	16.15	18.8	19.4
8	14.69	18.05	18.9	18.5
9	16.16	18.93	16.6	15.8
10	17.56	20.38	13.8	13.7
11	18.53	21.28	9.1	11.4
12	19.38	21.40	7.6	10.2
13	19.91	22.05	8.2	9.8
14	20.38	22.38	6.6	9.4
15	20.84	23.23	7.2	9.9
16	21.13	23.10	7.0	8.9
17	21.47	23.50	7.2	8.5
18	22.22	24.08	6.6	8.7
19	22.81	24.45	5.9	7.5
20	23.34	24.60	6.5	8.0
21	23.34	24.83	6.3	8.2
22	23.75	25.75	5.6	7.6
23	23.50	25.43	5.5	7.9
24	23.44	25.23	6.4	8.7
25	23.84	25.08	6.6	8.6
26	23.94	26.02	5.1	8.4
27	24.09	25.75	5.1	7.4
28	24.19	26.16	5.9	8.8
29	24.37	26.03	5.3	8.8
30	24.50	26.71	5.9	8.8
32	24.94	26.93	6.5	8.8
34	25.19	27.23	5.5	8.5
36	25.41	28.08	5.0	8.8
38	25.47	28.00	5.2	8.8
40	26.00	27.97	5.3	8.3
42	26.00	28.38	5.4	9.5
44	25.72	28.50	5.7	10.0
46	25.88	28.38	5.4	10.1
48	25.88	29.06	5.1	9.1
50	25.81	29.17	5.1	9.7
52	26.47	29.13	6.2	9.3
54	26.91	29.39	5.2	8.7

TABLE X—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	26.59	29.21	5.8	9.0
58	26.97	29.04	5.4	7.8
60	26.84	28.96	6.2	8.1
62	26.50	28.83	4.9	7.5
64	26.94	29.25	4.9	7.6
66	26.81	29.25	5.7	7.4
68	26.53	28.77	5.3	7.3
70	27.16	28.85	6.4	7.8
72	27.16	28.56	5.2	6.4
74	26.78	29.06	6.1	8.1
76	26.94	28.63	5.7	7.6
78	27.00	27.75	4.1	5.1
80	27.22	28.50	5.5	6.5
82	26.75	28.25	6.9	7.5
84	26.66	27.83	5.0	6.0
86	26.47	29.13	6.0	6.7
88	26.91	28.83	5.8	6.0
90	27.22	28.75	6.4	6.4
92	27.25	28.25	5.4	5.2
94	26.84	28.00	6.7	6.6
96	26.63	26.63	6.8	6.8

Summary of Table X.—The long lived animals are initially lighter than the short lived group. This relative subnormality is enhanced with time and amounts by the 40th week to 2.5 gm. They are consistently and very decidedly less variable than the short lived group. The short lived animals display a marked increase of weight between the 30th and 50th weeks, death being preceded by a gradual slight loss of weight.

TABLE XI.

Lecithin-Fed Females.

(Average duration of life 677 days.)

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
4	9.44	8.00	14.7	16.3
5	11.89	10.85	16.3	15.8
6	13.58	13.09	14.3	12.7
7	14.97	15.12	12.3	9.7
8	16.34	16.62	9.5	8.8
9	16.79	17.24	9.4	8.6
10	17.24	17.41	10.0	9.6
11	18.00	18.18	9.0	8.2
12	18.37	19.00	8.6	8.6
13	18.42	19.06	9.0	8.2
14	18.89	19.69	7.9	7.6
15	19.50	19.97	9.7	8.9
16	19.87	20.22	8.3	8.4
17	20.03	20.53	8.7	8.5
18	20.13	20.94	9.0	9.0
19	20.45	21.16	8.7	8.6
20	20.32	21.03	8.5	8.5
21	20.71	21.41	9.7	8.8
22	20.76	21.56	9.9	9.1
23	21.03	21.66	9.3	9.0
24	20.97	21.63	9.5	9.4
25	21.03	21.72	10.4	9.4
26	21.47	21.88	9.8	8.9
27	21.29	22.00	10.2	9.8
28	21.76	22.09	10.7	9.8
29	21.79	22.38	10.4	10.2
30	22.18	22.50	10.2	10.6
32	22.16	22.47	11.6	11.2
34	22.32	22.89	10.7	10.9
36	22.61	23.25	10.4	10.8
38	22.47	23.29	11.7	11.4
40	22.74	23.29	11.3	11.2
42	22.82	23.25	13.2	12.1
44	22.97	23.71	12.8	12.9
46	22.74	23.88	14.6	13.6
48	23.39	24.92	13.4	14.2
50	23.34	24.08	14.7	14.4
52	24.13	24.60	13.8	14.0
54	23.84	25.90	14.0	14.7

TABLE XI—*Concluded.*

Age.	Average weight of long lived animals.	Average weight of short lived animals.	Variability of long lived animals.	Variability of all animals combined.
wks.	*gm.*	*gm.*	*per cent*	*per cent*
56	23.63	25.50	14.2	14.1
58	24.00	26.00	13.9	13.5
60	23.87	24.56	14.3	13.1
62	23.39	24.44	13.4	12.4
64	24.26	24.50	14.6	12.9
66	24.84	24.78	14.0	13.1
68	23.74	24.11	15.4	14.4
70	24.53	24.81	14.8	14.4
72	24.13	20.50	14.3	15.0
74	24.29	25.17	15.0	14.2
76	24.66	25.50	15.3	14.5
78	24.71	26.33	15.2	14.3
80	24.45	26.50	15.4	14.4
82	24.39	26.33	15.3	14.4
84	23.97	26.25	15.0	14.5
86	24.08	26.50	16.6	15.8
88	24.24	27.25	16.3	15.7
90	24.47	26.00	18.0	17.6

Summary of Table XI.—The long lived animals are initially heavier and less variable than the short lived animals, but from 8 weeks onward the short lived animals are slightly heavier than the long lived animals, the variability of the two groups being the same.

TABLE XII.

Experimental group.	Duration of life.				Difference as per cent of average.
	Average.	Long lived.	Short lived.	Differ-ence.	
	days	*days*	*days*	*days*	*per cent*
Males:					
Tethelin	866	979	718	261	30
Pituitary	792	926	649	277	35
Normal	767	928	606	322	42
Cholesterol	764	889	592	297	39
Lecithin	731	943	519	424	58
Females:					
Tethelin	800	970	630	340	42
Normal	719	863	533	330	46
Pituitary	704	831	514	317	45
Tethelin (brief administration)	695	867	472	395	57
Lecithin	677	829	484	345	51
Cholesterol	658	830	486	344	52

SOME OBSERVATIONS ON COLORIMETRIC ESTIMATIONS WITH SOLUTIONS CONTAINING TWO COLORED SUBSTANCES.

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1. INTRODUCTION.

Colorimetric methods for the estimation of small quantities of various substances have been developed to a considerable extent in recent years. At present, such methods appear to offer the most promising future for the quantitative determination of certain constituents present in small amounts in blood. The advances in these methods have been remarkable, although at times their accuracy and reliability have been somewhat overestimated. The general principle of such methods involves the reaction of the constituent to be determined with an added substance to produce a colored substance in a definite chemical ratio, and the estimation of the amount of colored substance formed by colorimetric comparison with a known amount of standard substance. In such methods, a colored substance may be produced from a (to the eye) colorless substance, as in the uric acid method of Folin and Denis[1], or one colored substance may be converted into a differently colored substance. The latter method evidently suggests possibilities of confusing complexity. The glucose method of Lewis and Benedict[2] applied to blood and of Dehn and Hartmann[3] depends upon the production of a red to brown reduction product, probably picramate, from a yellow picrate under definite conditions. This method was developed further by Benedict and Osterberg[4] so as to make possible the determination of the glucose (or reducing substances) present in ordinary "normal" urines. Because of some apparently contradictory results obtained by different workers with this method, a careful study was made of simple conditions involved in this reaction. Certain phases of the results which are presented here are given in some detail because of their general interest and applicability to all colorimetric measurements.

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[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912–13 xiii, 469.

[2] Lewis, R. C., and Benedict, S. R., *Proc. Soc. Exp. Biol. and Med.*, 1913–14, xi, 57; *J. Biol. Chem.*, 1915, xx, 61.

[3] Dehn, W. M., and Hartmann, F. A., *J. Am. Chem. Soc.*, 1914, xxxvi, 403.

2. Significance of Colorimetric Comparisons.

Some space may be devoted to the physical significance of the readings obtained with colorimeters in general use. When two solutions are matched in these, they have the same color. They need not be identical in composition or content. A color, as determined by the eye, does not fix the wave-lengths in the spectrum given by the substance. It is frequently stated that the wave-lengths of the spectrum of a substance determine its color. This statement requires a significant reservation. If two different wave-lengths are shown in the spectrum of a substance, then the color of the substance, for example in solution by transmitted light, will have a certain value to the eye. By color value is meant the physiological sensation which is considered to be produced by color and intensity of illumination. It is difficult to form a mental picture of these two properties separately, but it can readily be understood that both are involved in color sensation. For want of a better term, color value is used here for this phenomenon. If the concentration of the substance in the solution is halved, then doubling the length of the column of solution would give the same color value to the eye, since the light passes through the same amount of colored substance. The assumed two wave-lengths of the spectrum possess certain intensities which take part in determining the color value observed. A second substance will now be taken which at a certain concentration in solution is matched in a colorimeter against the first. The spectrum of the second substance is assumed to give two wave-lengths differing from those of the first substance, but the colors of the two substances as seen by the eye are the same at certain concentrations. Varying the concentration of the second substance in solution will give comparable results when matched against itself, as does the first, but colorimetric comparisons of different concentrations of the two substances may give irregular results because the intensities of the different sets of wave-lengths may be different and show different variations on dilution. The color values are determined by both the wave-lengths and their intensities, so that variations in the latter may result in unexpected color values as determined by the eye for solutions which might be expected to be similar.

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[4] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

The hypothetical case just outlined is made actual by the colorimetric comparisons of bichromate and picramate solutions, the results of which are given in Section 5 *d*. It would appear that this relation applies and must be taken into account whenever two solutions containing differently colored substances are compared colorimetrically.

Identity of color as determined visually is the basis of the use of colorimeters such as the Duboscq. Such a color may be made up of two or more colored substances or of a pure substance, and still show visual identity with a different substance. This leads to another conclusion. Very few eyes are exactly similar in matching color, so that with mixed colored substances differences may be observed by different individuals. Such differences are less likely to occur with a substance of a definite color matched against itself in different concentrations. The colorimeter does not compare differences in color. When a match is obtained, the colors are assumed to be identical. The intensity of illumination of the two fields in a colorimeter gives a measure of the relative concentrations of the two solutions being compared. Very small differences in intensity of illumination, or amount of light per unit of surface, or brightness, can be observed. In practical work, the lengths of the columns of the liquids are varied until the fields are identical in intensity. The heights of the columns through which the light passes give then the relative concentrations of the dissolved substance in the two solutions. The light in the two cases is assumed to pass through the same absolute amounts of colored substance. This assumes Beer's law, that the amount of absorption is proportional to the concentration of dissolved substance. Comparing different concentrations of a solution of one colored substance, this law holds in all probability for the ordinary uses to which a colorimeter is put. It is obvious, however, that complications may result as a consequence of assuming the validity of this law when comparing solutions of the same visual color at various concentrations, or when the colors are due to different substances or to the (visual) combination of two colored substances in varying proportions in the different solutions which are compared. Also the nature of the source of light may influence colorimetric readings, so that the exact illumination used should be stated in every colorimetric study.

3. Experimental Conditions in the Benedict Method.

After removal of interfering substances from the urine, or similar solution, Benedict and Osterberg determine the glucose, or reducing substance, by the partial reduction by heat in a carbonate solution of a yellow picrate to a red product, using as a standard for color comparison a glucose solution treated similarly or a picrate-picramate solution, or if picramate is not available a bichromate solution. The variable factors in the unknown solutions compared colorimetrically are the concentrations of picrate, of alkali, and of picramate. The lengths of time of heating to develop the colors may also be varied. In the final unknown solutions, the content of picrate (calculated as picric acid) is 5.8 mg. or 11.5 mg. per cc., the latter with the more dilute glucose solutions, the former with the more concentrated. These figures are not corrected for the amounts of picrate reduced, but these amounts are negligible. The amount of sodium carbonate added to bring about the reaction to produce the color is the same in every case, but before colorimetric comparison the solutions are diluted with water so that finally the concentrations of the sodium carbonate added may be in the ratio 2 to 1 in different solutions. The concentration of picramate or reduced substance will obviously vary with the concentration of glucose or reducing substances. As for the time of heating, it was originally stated that 10 minutes were satisfactory. It was found later that this was insufficient, but the most satisfactory length of time was not fixed. Greatly as a result of the color comparisons, Benedict considered the production of picramate to be the reaction taking place.

4. Experimental Method.

A Duboscq colorimeter with cups 52 mm. deep and 20 mm. inside diameter, and plungers 113 mm. in length was used in this work. The colorimeter was enclosed in a box the inside walls of which were painted with white paint. A National Mazda (Western Electric Sunbeam) 75 watt, 120 volt lamp, the direct rays of which were screened from the colorimeter by a partition, was used. Indirect constant illumination was obtained in this way. Heating was avoided by having a slit in the rear of the box for ventilation and by cutting off illumination when readings were not being made. All the precautions ordinarily observed in the use of a colorimeter were taken. Every result given was the average of from four to eight readings.

The picric acid used was purified and tested by the method of Folin and Doisy.[5] With a saturated solution of picric acid set at 20 mm. in the colorimeter, an average reading of 13.3 mm. was given by 20 cc. of this solution to which 1 cc. of 10 per cent sodium hydroxide solution had been added and allowed to stand for 15 minutes. After 24 hours, no change could be detected.

Picramic acid was prepared from picric acid and ammonium sulfide, and crystallized from acetic acid and then from water. It melted sharply at 168° (corrected).

The glucose used was the purest commercially obtainable and was dried before being used. Made up to a 4 per cent solution, it showed a rotation of 4.14° in a 200 mm. tube at 25° with sodium light (calculated 4.20°).

5. Experimental Results.

In order to obtain as complete a picture as possible of the conditions involved in the picrate-sugar method, the following series of experiments were carried out:

(*a*) Picrate against picrate (varying concentrations and alkalinities).

(*b*) Bichromate against bichromate (varying concentrations).

(*c*) Picramate against picramate (varying concentrations and alkalinities).

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[5] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 352.

(*d*) Picramate against bichromate.

(*e*) Picramate plus picrate against picramate plus picrate (varying concentrations).

(*f*) Glucose-picrate product against picramate plus picrate (varying concentrations).

(*a*) *Picrate against Picrate* (*Varying Concentrations and Alkalinities*).—The colors of picric acid solutions, even when saturated, were too faint for colorimetric study. The color of a picrate solution deepens with increase in concentration of sodium hydroxide as pointed out by Folin.[6]

With a solution to which had been added equivalent amounts of picric acid and sodium hydroxide matched against a similar solution as standard, addition of sodium hydroxide deepened the color, but addition of sodium carbonate did not until after a certain quantity had been added. For example, in a solution which contained per cc. (calculated in terms of the constituents added)[7] 14.4 mg. of picric acid and 2.5 mg. of NaOH, practically no change in color was observed by the addition of sodium carbonate until more than 9.6 mg. per cc. (or almost three equivalents per equivalent of picric acid) had been added.

The concentrations in the solutions used by Benedict are well within these limits. The picrate solution used in the development of the color contains approximately 36 mg. of picric acid per cc.; including the alkali, picric acid, and sodium hydroxide in the ratio of equivalents of nearly 1.25 to 1. The sodium carbonate added makes the final solutions, omitting the very small amount of picrate reduced in the reaction, have the following content per cc. when compared in the colorimeter:

When made up to a final volume of 25 cc.:
5.8 mg. of picric acid; 0.8 mg. of NaOH; 8.0 mg. of Na_2CO_3.
When made up to a final volume of 12.5 cc.:
11.5 mg. of picric acid; 1.6 mg. of NaOH; 16.0 mg. of Na_2CO_3.

(*b*) *Bichromate against Bichromate* (*Varying Concentrations*).—Accurate estimations of the content of potassium bichromate in solution may be made by colorimetric comparisons. For example, halving the concentration was found to give twice the colorimeter reading at different settings.

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[6] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475. Hunter A., and Campbell, W. R., *J. Biol. Chem.*, 1916–17, xxviii, 336.

[7] This method of stating the contents of a solution will be followed throughout this paper.

(*c*) *Picramate against Picramate* (*Varying Concentrations and Alkalinities*).—Some experiments were performed with solutions of picramic acid, in which different concentrations were compared colorimetrically. The color values so determined were not proportional to the concentration of picramic acid. Also, exposure to light deepened the color of a picramic acid solution. Since picramic acid as such is not used in the method under investigation, these observations were not continued.

A number of solutions of picramate with different quantities of excess alkali were compared. Each solution was made up by dissolving 0.050 gm. of picramic acid in 500 cc. of the appropriate solution. Table I shows the results obtained when each was compared with all the others successively. 1 cc. of every solution contained 0.1 mg. or 0.0005 milli-equivalents of picramic acid.

TABLE I.

Content.		Found.
Na_2CO_3		Picramic acid per cc.
per cent	*milli-equivalents per cc.*	*mg.*
0.8	150	0.098 (0.092–0.100)
1.6	300	0.098 (0.092–0.101)
3.2	600	0.098 (0.093–0.100)
6.4	1,200	0.100 (0.093–0.104)
NaOH		
2.5	625	0.104 (0.100–0.108)
5.0	1,250	0.104 (0.100–0.109)

The results in parentheses indicate the extremes of the averages of the comparison of the separate series with increasing alkalinity. With the carbonate solutions, the concentration of carbonate had no influence on the colorimetric comparisons. With sodium hydroxide solutions, there was apparently a slight increase in color, but not enough to introduce appreciable error. These alkaline solutions, exposed to the electric light for 24 hours, showed no difference in color, in contradistinction to the picramic acid solutions.

A strict proportionality was also found to exist between the colorimetric readings and concentration of alkaline picramate solutions with the sodium carbonate content recommended by Benedict and Osterberg.

(*d*) *Picramate against Bichromate.*—It was shown in Sections *b* and *c* that the color values, as determined colorimetrically, of bichromate solutions were proportional to the concentrations over wide ranges, and, similarly, that the color values of picramate solutions were proportional to the concentrations over wide ranges.

A number of bichromate and picramate solutions were compared colorimetrically. Table II shows some of the results of such a series.

TABLE II.

Bichromate.				Picramate Solution B.	
Solution A.		Solution B.			
Concentration per cc.	Colorimeter readings.	Concentration per cc.	Colorimeter readings.	Concentration as acid per cc.	Colorimeter readings.
mg.	*mm.*	*mg.*	*mm.*	*mg.*	*mm.*
15.0	15.0	15.0	15.0	0.042	18.5 (1)
15.0	15.0	11.3	20.1	0.031	24.6 (2)
15.0	30.0	11.3	40.1	0.042	26.3 (3)
11.3	15.0	15.0	10.9	0.031	21.0 (4)

Bichromate Solution A was used as the standard. The readings with Solution B were found to be inversely proportional to the bichromate concentrations at different settings. Readings 1 and 2 of the picramate solution were inversely proportional to the picramate concentrations with the same concentration and depth of bichromate solution. If proportionality to concentration held, Reading 3 of picramate solution should have been twice that of Reading 1 or 37.0, since the depth of the bichromate was twice as great. The value found was 26.3. From Readings 1 and 2, Reading 4 for picramate solution should have been 18.5. The value found was 21.0.

A number of series of results were obtained showing essentially the same relations as those given with greater or less variations depending upon the concentrations of bichromate and picramate. Since Benedict and Osterberg suggest the use of bichromate as standard only if the other standards are not available, these results will not be given in detail. This difference in the color match is perceived much more readily by some eyes than by others. If the eye is very sensitive to such differences, the results will be correspondingly less accurate. This difference is also brought out clearly by inserting glasses of different colors, such as green, in the colorimeter so that the light before it reaches the eye passes through them. However, it is possible to obtain a match in most cases, but a greater latitude must be allowed here because of this difference.

The conclusions from the results may be stated as follows:

1. With a bichromate solution of unvarying concentration as a standard, always set at the same height in the colorimeter, solutions of picramate give readings inversely proportional to their concentrations.

2. Read against bichromate solutions of different dilutions set at the same height, or against the same bichromate solution set at different heights, picramate solutions do not give proportional readings.

The use of bichromate as standard depends, therefore, upon the determination of the colorimetric equivalence of bichromate and picramate for the definite colorimeter setting and concentration of the bichromate solution used.

Folin pointed out this same fact, in his paper on the determination of creatinine, using a bichromate solution as standard.[8] He has since abandoned bichromate in favor of creatinine itself.[9]

The differences pointed out apply in general to colorimetric comparisons of solutions of substances whose colors are visually alike but due to different absorption spectra. It was pointed out by Newcomer[10] that "It is thus essential in order that a color match be satisfactory under varying conditions that the absorption curves of the two substances composing the match be as near alike as possible." In any other case, the limitations of standard solution setting and concentration must be adhered to.

(*e*) *Picramate Plus Picrate against Picramate Plus Picrate* (*Varying Concentrations*).—Since the picrate sugar method involves the use of two colored substances, it is of interest to compare the color values of these substances. An attempt was therefore made to determine the mg. of picrate equivalent colorimetrically to 1 mg. of picramate. A number of methods may be used based upon suitable colorimetric measurements.

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[8] Folin, O., *Z. physiol. Chem.*, 1904, xli, 223.

[9] Folin, O., *J. Biol. Chem.*, 1914, xvii, 469.

[10] Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 486.

When two solutions match (possess the same color value), the ratio of their color values per cc., or per mm. of height as determined in a colorimeter, is inversely proportional to these heights. Assuming proportionality of concentration to color value within a limited range, with one solution as standard, for the other solution the concentrations of picrate and picramate may be calculated which will give the same color value as the first solution. From these, the colorimetric ratio of picramate to picrate can readily be calculated.

The general relations involved may be expressed as follows:

P = Color value of 1 mg. of picramic acid (present as picramate).
p = " " " 1 " " picric " (" " picrate).
R_s = Reading of height of standard solution in colorimeter.
R_u = " " " " unknown " " "
s = Mg. of picramic acid (present as picramate) per cc. in standard solution.
s' = Mg. of picric acid (present as picrate) per cc. in standard solution.
u = Mg. of picramic acid (present as picramate) per cc. in unknown solution.
u' = Mg. of picric acid (present as picrate) per cc. in unknown solution.

For the same color value of the standard and unknown

$$sP + s'p = \frac{R_u}{R_s}\,(uP + u'p)$$

From this it follows that the ratio of the color values of the same weights of picramic and picric acids (present as their salts in alkaline solution) is given by the expression

$$\frac{P}{p} = \frac{u'\,\dfrac{R_u}{R_s} - s'}{s - u\,\dfrac{R_u}{R_s}}$$

Also, if the ratio $\frac{P}{p}$ is known, then, comparing two solutions both of which contain known concentrations of picrate, and one a standard amount of picramate, the concentration of picramate (calculated as picramic acid) in the unknown is given by the expression

$$u = \frac{R_s}{R_u}\left(s + s'\frac{p}{P}\right) - u'\frac{p}{P}$$

In the following experiments, the picrate and picramate are always expressed as mg. of picric and picramic acids per cc. of solution. Enough excess alkali is present in every case to bring out the necessary color as found in preceding sections.

In order to obtain the value of the color equivalence of picramate and picrate, series of experiments were carried out in which colorimetric comparisons were made between picramate on the one side and picramate plus picrate on the other; between picramate plus a small amount of picrate (having a color value very close to the solution recommended by Benedict) on the one side and picramate and picrate on the other; and between picramate and picrate on both sides. Table III shows some of the typical results obtained. Solutions A and B refer to the two being compared, and concentrations are given in the usual terms. The actual readings obtained are not given. These were varied over considerable ranges, but regularities connected with these variations were not observed. The color value ratios of picramate to picrate were calculated by means of the equation given in the table.

The first striking fact of these results is their apparent lack of constancy for the picramate-picrate ratio. This may be only apparent to some extent, as it was found in some cases that small differences in readings would cause comparatively large differences in the calculated ratios.

For example, a difference in the colorimeter readings of 0.3 mm. each would have resulted in the ratio of 1,000:1 for the seventh, tenth, and thirty-second results where the ratios found were 750, 1,100, and 1,210 to 1. Similarly, a difference in the reading of 0.4 mm. for the first result and 0.6 for the fifth, where the found ratios were 440 and 810 to 1 would have given the ratio

TABLE III.

Solution A.		Solution B.		Color ratio picramate: picrate.
Concentration of picrate.	Concentration of picramate.	Concentration of picrate.	Concentration of picramate.	
mg. per cc.	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	
	0.080	7.2	0.080	440
	0.070	10.8	0.070	550
	0.060	14.4	0.060	650
	0.050	18.0	0.050	800
	0.040	21.6	0.040	810
	0.030	25.2	0.030	950
	0.035	5.4	0.035	750
	0.031	9.0	0.031	910
	0.027	12.6	0.027	950
	0.023	16.2	0.023	1,100
	0.019	19.8	0.019	980
	0.100	7.2	0.080	450
	0.100	10.8	0.070	590
	0.100	14.4	0.060	640
	0.100	18.0	0.050	780
	0.100	21.6	0.040	860
	0.100	25.2	0.030	840
	0.042	5.8	0.015	1,060
	0.042	5.8	0.018	1,060
	0.042	5.8	0.022	860
	0.042	5.8	0.025	910
	0.042	5.8	0.028	890
	0.042	5.8	0.032	790
(2.0)	0.042	5.8	0.015	1,140
(2.0)	0.042	5.8	0.018	1,070
(2.0)	0.042	5.8	0.022	1,130
(2.0)	0.042	5.8	0.025	910
(2.0)	0.042	5.8	0.028	890
(2.0)	0.042	5.8	0.032	1,080
(2.0)	0.042	5.8	0.036	1,190
(2.0)	0.042	5.8	0.040	1,390
(2.0)	0.042	5.8	0.044	1,210
(2.0)	0.042	5.8	0.048	990
(2.0)	0.042	11.5	0.015	1,340
(2.0)	0.042	11.5	0.018	1,270
(2.0)	0.042	11.5	0.022	1,320
(2.0)	0.042	11.5	0.025	1,180
(2.0)	0.042	11.5	0.028	1,220
(2.0)	0.042	11.5	0.032	1,120

TABLE III—*Concluded.*

Solution A.		Solution B.		Color ratio picramate: picrate.
Concentration of picrate.	Concentration of picramate.	Concentration of picrate.	Concentration of picramate.	
mg. per cc.	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	
(2.0)	0.042	11.5	0.036	1,010
(2.0)	0.042	11.5	0.040	950
(2.0)	0.042	11.5	0.044	910
(2.0)	0.042	11.5	0.048	810
5.8	0.032	5.8	0.016	790
5.8	0.048	5.8	0.016	1,040
5.8	0.064	5.8	0.016	920
5.8	0.048	5.8	0.032	1,310
5.8	0.064	5.8	0.032	1,180
5.8	0.064	5.8	0.048	1,220

of 1,000: 1. These results give perhaps the extreme variations for difference in ratio with small difference in colorimeter reading. The variation of the ratio is evidently connected with the differences in concentration of both picrate and picramate in both solutions. With four such variables to deal with, it is impossible to draw general conclusions with regard to the change with the results obtained.

The first twenty-three results were obtained by comparison with pure picramate solutions (containing excess of alkali). In the Benedict method, a standard solution containing picramate and picrate is prepared very nearly equivalent to the color developed by 1.0 mg. of glucose with the 4 cc. of picrate solution, heated for 10 minutes, finally diluted to 25 cc. Solution A with 0.042 mg. of picramic acid (as picramate) per cc. has a color value equivalent to that produced by this amount of glucose when the heating was continued for 1 hour. The next twenty experiments had for Solution A a color value equivalent to 0.042 mg. of picramic acid per cc. as before, but made up with the relative concentrations of picrate and picramate used by Benedict as standard. This contained very nearly 2.0 mg. of picric acid (as picrate) per cc., and a colorimetric equivalent less of the picramate. This is shown in the table by placing the 2.0 mg. of picric acid in parentheses.

Forty-nine results are given. Thirty-one of these give picramate-picrate color ratios between 800 and 1,200 to 1. Ten are below 800, of these two of 790 each, one of 780. Eight are above 1,200, of these one of 1,210, two of 1,220. The average of the forty-nine results gives a ratio of 963 to 1. The average of the thirty-one results (800 to 1,200) gives 984. Of the four results giving ratios less than 600 to 1, two were obtained with 0.080 mg. of picramic acid per cc., and two with 0.070 mg. of picramic acid per cc. Some experiments with greater concentrations of picramic acid gave still smaller ratios. These decreasing ratios with increasing picramate are evidently to be referred to the same cause as the lack of equivalence of bichromate and picramate solutions at different concentrations described in Section 4 *d*. The color values as determined by the eye vary differently in these solutions containing relatively more picramate than where small amounts are present.

In view of the possibility of experimental inaccuracy, and also because the ratio serves in the main as a correction term, the round value of 1,000 to 1 will be used here as the color value equivalent of picramic and picric acids (present as their salts). In solutions containing considerably larger concentrations of picramate than are met with in general work, this ratio will be less; if considerably less picramate is in solution than is found ordinarily, this ratio must be increased.

As stated before, this varying ratio of colorimetric equivalence of picramate and picrate probably is to be referred to the physical method of determining color values and not to any chemical change in the substances in solution. It is also probable that the ratio of the concentrations of picramate and picrate in the solutions is the predominating factor which determines their colorimetric equivalence, although, because of the measurements which were carried out, greater emphasis was placed upon the picramate concentrations given in the tables. That is to say, for high concentrations of picramate and low of picrate, 1 mg. of picramic acid (present as picramate) will possess a color value equivalent to less than 500 mg. of picric acid (present as picrate), possibly as low as 200 mg. in some cases. For low concentrations of picramate and high of picrate, 1 mg. of picramic acid (present as picramate) will be equivalent in color value to 1,500 mg. or more of picric acid (present as picrate). For intermediate concentrations as shown in the table, the ratio is in the neighborhood of 1 to 1,000.

This lack of constancy in the equivalence of the color values of picramate and picrate obviously causes a measure of uncertainty in correcting for the presence of picrate in the colorimetric measurements. This is true especially when the solutions which are being compared contain widely different concentrations of the two constituents.

(f) Glucose-Picrate Product against Picramate Plus Picrate (Varying Concentrations.—In the preceding sections an attempt was made to describe the relations which appear to underlie the colorimetric comparisons of the glucose-picrate method. There will be presented in this section some of the results and conclusions obtained with glucose solutions bearing more directly upon the determination of glucose as described by Benedict and Osterberg.

Benedict and Osterberg used various solutions as their standards. In the first place they suggest a solution prepared from glucose at the same time and in the same way that the filtrates of the unknown solution are treated. Such a solution would contain 5.8 mg. of picric acid (as picrate) and colored substance obtained from 0.04 mg. of glucose per cc. The best permanent standard (giving a color value very nearly that given by the picrate solution with 0.04 mg. of glucose) as given by them contained very nearly 1.8 mg. of picric acid and 0.35 mg. of picramic acid (both present as their salts) per cc. Where pure picramic acid is not available, they suggested the use of potassium bichromate as a standard but indicated that the other standards are preferable.

Some points in their experimental procedure may be discussed briefly. Since they found that increasing the concentration of sodium carbonate in the glucose solutions produced continuously increasing color, the concentration recommended by them as best was adhered to strictly in this work. The length of time of heating the glucose solution to produce the colored substance is an important question. In order to obtain quantitative and comparable results in every case, it would appear that a definite reaction must take place leading to definite products. This implies that an end-point must be reached which is stable to a certain extent, so that further treatment within reasonable limits will not produce further change. Benedict and Osterberg stated in their paper that 10 minutes heating sufficed, but in a private communication expressed the opinion that a longer time was advisable.

A number of series of experiments were carried out to test this view. Table IV gives some of these results. The solutions as read contained 0.040 mg. of glucose, 5.8 mg. of picric acid, and the usual concentration of alkali per cc. The procedure of Benedict and Osterberg was followed strictly, except for the different times of heating. Comparisons were made with the standard picramate solution recommended by Benedict and Osterberg.

TABLE IV.

Time of heating.	Colorimetric readings.		
	Solution I.	Solution II.	Solution III.
min.			
5	14.4	17.6	22.6
10	13.6	15.4	17.7
15	13.6	14.9	15.8
20	13.2	15.1	15.2
25	13.5	14.6	15.3
30		14.4	15.1
35		14.7	14.9
40		14.5	14.7
45		14.5	14.5
50		14.1	14.2
55		14.4	14.3
60		14.2	13.9

With Solutions I and II, the color was developed directly with the glucose solution; Solution III was submitted to the precipitation procedure with mercury salt and alkali before the color was developed.

With the salts from the precipitation procedure present, considerably longer time was needed for full color development. With pure glucose solution 10 to 20 minutes appear to be sufficient. There was practically no further change here on heating to 1 hour. Various factors influence the rate of production of colored substance from the picrate, such as alkalinity of solution, concentration of glucose, and the presence of other substances.

Benedict[11] emphasized the fact that very small amounts of acetic acid in excess lead to a remarkable decrease in the rate of color development, while Okey[12] pointed out the retarding effect of sodium chloride.

In view of these facts, and because it was considered inexpedient to heat for a short period of time on the assumption that the reaction had proceeded to a certain definite point, it was decided to heat all glucose solutions for 1 hour in studying this reaction. This length of time of heating results in a difference in the concentration of the permanent standard recommended by Benedict and Osterberg and prepared by color comparison with a solution from 0.04 mg. of glucose per cc. They found that a solution containing 1.8 mg. of picric acid and 0.035 mg. of picramic acid (present as their salts) gave a satisfactory standard for this amount of glucose. For the longer time of heating, and using the constituents in the solution in the same ratio that they did, it was found that the concentrations giving a color value equivalent to this amount of glucose contained very nearly 2.0 mg. of picric acid and 0.040 mg. of picramic acid (present as their salts) per cc.

The results obtained are given in Table V. The glucose solutions were treated with the sodium carbonate solutions recommended by Benedict and Osterberg, the volumes of glucose solution ranging from 1.0 to 4.0 cc. and the total glucose content from 0.42 to 1.5 mg., the concentrations given in the table representing mg. of glucose per cc. after dilution. These were heated for 1 hour, then diluted with water to 25 cc. or 12.5 cc., and compared with the standard solution of picrate plus picramate. The usual excess of alkali was present.

Columns 1 and 2 show the concentrations of picric acid and picramic acid (present as their salts) in the standard, Column 3 the concentration of picric acid (present as its salt) in the "unknown," and Column 4 the added concentration of glucose in the "unknown" solution. From the colorimetric readings, the concentration of picramate in the unknown was calculated, correction for the picrate present being made on the assumption that the colorimetric equivalents of the color values of picramate to picrate were in the ratio of 1,000 to 1. From these values and

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[11] Benedict, S. R., *J. Biol. Chem.*, 1919, xxxvii, 503.

[12] Okey R., *J. Biol. Chem.*, 1919, xxxviii, 33.

TABLE V.

Standard.		Unknown.		Color ratio glucose:picramic.
Picric.	Picramic.	Picric.	Glucose.	
mg.	*mg.*	*mg.*	*mg.*	
5.8	0.016	5.8	0.034	1.44
5.8	0.016	5.8	0.038	1.38
5.8	0.016	5.8	0.040	1.36
5.8	0.016	5.8	0.042	1.29
5.8	0.016	5.8	0.054	1.26
5.8	0.032	5.8	0.034	1.36
5.8	0.032	5.8	0.038	1.31
5.8	0.032	5.8	0.040	1.29
5.8	0.032	5.8	0.042	1.26
5.8	0.032	5.8	0.054	1.19
5.8	0.048	5.8	0.034	1.38
5.8	0.048	5.8	0.038	1.32
5.8	0.048	5.8	0.040	1.28
5.8	0.048	5.8	0.042	1.29
5.8	0.048	5.8	0.054	1.20
5.8	0.064	5.8	0.034	1.35
5.8	0.064	5.8	0.038	1.30
5.8	0.064	5.8	0.040	1.28
5.8	0.064	5.8	0.042	1.23
5.8	0.064	5.8	0.054	1.17
5.8	0.020	5.8	0.034	1.33
5.8	0.020	5.8	0.038	1.31
5.8	0.020	5.8	0.040	1.32
5.8	0.020	5.8	0.042	1.30
5.8	0.020	5.8	0.054	1.20
5.8	0.040	5.8	0.034	1.26
5.8	0.040	5.8	0.038	1.32
5.8	0.040	5.8	0.040	1.26
5.8	0.040	5.8	0.042	1.20
5.8	0.040	5.8	0.054	1.17
Mean..				1.287
5.8	0.016	11.5	0.034	2.06
5.8	0.020	11.5	0.034	1.75
5.8	0.032	11.5	0.034	1.92
5.8	0.040	11.5	0.034	1.84
5.8	0.048	11.5	0.034	1.80
5.8	0.064	11.5	0.034	1.79

TABLE V—*Concluded.*

Standard.		Unknown.		Color ratio glucose: picramic.
Picric.	Picramic.	Picric.	Glucose.	
mg.	*mg.*	*mg.*	*mg.*	
11.5	0	5.8	0.034	1.63
11.5	0	5.8	0.038	1.54
11.5	0	5.8	0.040	1.53
11.5	0	5.8	0.042	1.45
11.5	0	5.8	0.054	1.43
11.5	0.030	5.8	0.060	1.17
11.5	0.030	5.8	0.050	1.25
11.5	0.030	5.8	0.040	1.32
11.5	0	11.5	0.034	2.18
11.5	0.030	11.5	0.060	1.39
11.5	0.030	11.5	0.050	1.58
1.8	0.035	5.8	0.060	1.10
1.8	0.035	5.8	0.050	1.15
1.8	0.035	5.8	0.040	1.20
1.8	0.035	5.8	0.030	1.57
1.8	0.035	5.8	0.020	1.87
1.8	0.035	11.5	0.060	1.32
1.8	0.035	11.5	0.050	1.46
1.8	0.035	11.5	0.040	1.76
1.8	0.035	11.5	0.030	2.10
1.8	0.035	11.5	0.020	5.40

the concentrations of glucose given in Column 4, the ratios of the equivalents of color values of glucose to picramic acid (present as its salt) for equal weights of the two were calculated and are given in Column 5.

The striking fact observed with the glucose-picramic ratio is its lack of constancy. Two immediate assumptions are introduced in the calculations; namely, the value and constancy of the picramic-picric ratio, and the definite character of the reaction by which glucose is transformed into the colored substance. This reaction is assumed to lead solely to the formation of picramate. It is probable that while picramate is the main product of the reaction other substances, even if only in very small amounts, are produced as well.

With regard to the constancy of the picramate-picrate ratio assumed, any other constant values would give similar irregular results for the glucose-picramic ratio. Only by assuming different ratios for different amounts of picramic and picric acids could constant results be obtained. Such ratios would be dependent in part upon the concentrations of the two substances both in the standard and in the unknown solutions.

These general irregularities are due undoubtedly mainly to the optical properties of the solution and the errors inherent in the visual method of comparison described in the preceding sections. At the same time, it is possible that the times of heating and the concentration of the alkali during heating might influence the results.

The results may be treated somewhat differently. Two readings with different solutions may be combined in a pair of simultaneous equations, with the picrate-picramate ratio and the glucose-picrate ratio as the unknown quantities. Solving a number of these sets of equations which involve no assumption as to the color equivalence of picrate and picramate gave similar varying values for the two ratios, indicating that the variations are inherent in the nature of the results (or the experiments).

In spite of this lack of constancy, it may be possible to go somewhat farther with these results. The optical properties might be expected to cause the smallest interference if the solutions compared are most nearly alike in composition. This similarity is evidently obtained with the first thirty results in Table V. The mean value of the glucose-picramic ratio is 1.29 to 1 for these. This indicates a fairly definite chemical reaction, which it would be possible to formulate if more chemical evidence were available.

The remaining results given in the table show greater variations in the glucose-picramic ratio, but also contain much greater differences in the contents of the solutions being compared. As stated before, although it is possible that the chemical reaction leading to the production of colored substance by the action of glucose on picrate may be different depending upon the concentration of the glucose and the more or less accidental presence of other substances, it is more probable that this ratio is much more constant than the results would indicate and that the apparent inconstancy is caused by the inconstant picramic-picric ratio and the optical properties of the solutions being compared.

At the same time, it may be pointed out that the percentage of the total color which is due to the glucose is large. It may vary from 60 per cent for very dilute glucose solutions to 80 and even 90 per cent for the solutions met with ordinarily.

Benedict and Osterberg did not enter into these questions specifically in their paper. However, from the results of the permanent standard given by them, the ratio of the color values of glucose to picramic acid (present as picramate) is found to be 1.14 to 1. This ratio is less than that of the average of a number of the results given in Table V. It is uncorrected for picrate color and is based upon 10 minutes heating of the glucose solution.

It is evident that Benedict and Osterberg developed conditions involving concentration of picrate, concentration of alkali, time of heating, *etc.*, which permitted them to obtain results of a considerable degree of accuracy in the determination of glucose. The extension of their work, for which the results are presented here, indicates that the conditions which they realized are limited, especially with regard to the concentration of glucose which may be estimated accurately. Especially with very small amounts of reducing substances present, where the standard and unknown solutions differ considerably in content and concentration might less accurate results be expected.

Because of the factors which may be varied in the standard and in the unknown, no attempt will be made here to show the limits of accuracy of the determinations with different amounts of glucose.

CONCLUSIONS.

An attempt was made to outline some of the principles underlying the ordinary use of the colorimeter for quantitative work. In connection with colorimetric estimations in mixtures of two colored substances, the glucose-picrate colorimetric method developed in detail by Benedict and coworkers was studied. It was pointed out that the optical properties as determined visually with a colorimeter permitted of accurate estimations for such solutions only in special cases. In order to be certain of quantitative results, the standard and unknown solutions, containing two colored substances, should be very nearly alike in composition and concentration. The errors introduced otherwise may be considerable. The experimental results leading to these conclusions are given in some detail for mixtures of picrate and picramate, and for mixtures of picrate and the reduction product of picrate obtained by the action of glucose.

It is desired to thank Dr. I. Greenwald for his invaluable aid in planning the experiments and in the treatment of the results. Thanks are also due Mr. R. P. Greene for continued help in the different parts of the investigation.

FAT-SOLUBLE VITAMINE.*

VI. THE EXTRACTABILITY OF THE FAT-SOLUBLE VITAMINE FROM CARROTS, ALFALFA, AND YELLOW CORN BY FAT SOLVENTS.

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During the course of the last few years, as fairly accurate quantitative data—that is quantitative in a biological sense—on the distribution of the fat-soluble vitamine in various plant materials had become available, it appeared increasingly desirable to attempt to concentrate this dietary essential for a study of its chemical properties. For some time we hesitated to initiate experiments of this nature as from our experience with the fat-soluble vitamine content of butter fat we were impressed with its instability under certain environmental conditions. Our attempts were therefore first directed to a study of its distribution (1, 2) and its stability under the conditions in which it is found in plant materials (3). While this work was far from being completed when these experiments were started our results were sufficiently inclusive to indicate that the fat-soluble vitamine as found in plants was very stable and that no immediate serious difficulty was to be anticipated even if no special precautions were taken to minimize its destruction.

The first experiments inaugurated were based upon our recently observed relations in the distribution of the fat-soluble vitamine in roots and maize with respect to the occurrence of certain yellow plant pigments (4). In the highly pigmented yellow roots such as yellow carrots and sweet potatoes there had been found an abundance of the vitamine as contrasted with the non-pigmented roots in which it was found absent or at most present in very small amounts. The same relations held with respect to maize. Out of four varieties of white maize extensively grown in the corn belt not a single one was found to contain appreciable amounts of the vitamine; yellow varieties of maize, on the other hand, never failed to give evidence of containing it in considerable quantities. It will be recalled that there are similar relations in the case of fats which have already been briefly touched upon (5), but which will be more extensively discussed in a later publication.

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As the fat-soluble vitamine was originally discovered in butter fat (6, 7) the one property available for its extraction and its accumulation which naturally would suggest itself to us was its solubility in fats. But here it was very obvious that butter fat by reason of its prohibitive cost could not be used in extensive preparations as a source of this dietary essential. It was, however, hoped that plant materials rich in the vitamine might yield it to fats by appropriate treatment.

For experiments of this nature both green leafy materials and carrots were available. It was planned to saturate these with lard, olive oil, or corn oil, extract the fat with ether, and then incorporate the extract in a ration for the determination of the vitamine. Preliminary experiments with olive oil and alfalfa yielded us an extract containing chlorophyll which proved impotent as a source of the vitamine. As chlorophyll in itself obviously was a constituent not to be desired in the extract we gave up the use of alfalfa and turned our attention to carrots on which we used corn oil and ether as the solvents in one case and lard and ether in the other. Our results were entirely unsatisfactory; a lard-ether extract gave absolutely negative results and a corn oil-ether extract gave results indicating that only traces of the vitamine were contained in it.

We next turned our attention to the direct extraction of the vitamine by means of fat solvents. Desiring to avoid the contamination of our extracts with chlorophyll and the large amounts of waxes associated with the external surfaces of plant structures, we concluded to use carrots in these experiments. Moreover, we surmised that extracts of carrots by virtue of the palatability of the roots would not prove distasteful to the animals. In this we were in error as the essential oils of the carrot in concentrated form are distinctly bitter. This proved a difficulty in our work, especially as our extracts were found so low in their content of the fat-soluble vitamine that it was found necessary to feed them in considerable concentration to obtain unequivocal demonstration of the presence of the vitamine. Ether gave entirely negative results, but alcohol, carbon disulfide, chloroform, and benzene gave fairly satisfactory evidence of solvent properties for the vitamine. In no case was the result entirely satisfactory as the extracted carrot residues when fed in small amounts as the sole source of the fat-soluble vitamine gave no evidence of having been seriously depleted in their vitamine content. It was evident to us that, while carrots contained the fat-soluble vitamine in abundant amounts, for some reason or other—probably on account of the presence of the colloidal pectins and hemicelluloses or possibly on account of the occurrence of the vitamine in molecular combinations—no satisfactory quantities of the vitamine could be accumulated from them. We then directed our attention to alfalfa again as our single failure to secure a potent fat extract with this material appeared no less promising than our results obtained with the same technique on carrots. We did not feel assured that fat solvents by themselves would not be efficacious in giving us active preparations, a surmisal which proved entirely justifiable as shown by our experimental results. Benzene and cold 95 per cent alcohol were found to be excellent solvents; ether gave far inferior results. With yellow maize, which was later added to the materials investigated, ether again gave poor results, but hot alcohol removed the vitamine almost quantitatively.

The apparent difficulties anticipated in connection with the production of a concentrated fat-soluble vitamine preparation now no longer appeared insurmountable. It remained to be seen whether our observations of the association in nature of plant pigments of the carotin and xanthophyll type, the so called carotinoids, would aid us in formulating additional experiments for the characterization of the vitamine. We had already found in numerous trials that the fat-soluble vitamine was not extracted by water which together with its demonstrated solubility in fat solvents made it appear highly probable that we were dealing with a lipoidal molecule. As the carotinoids are obtained by ether extraction of a saponified alcoholic extract of leafy materials we applied such a procedure in an attempt to secure an active preparation from alfalfa. In this we were entirely successful. Furthermore, a fractionation of this material by the methods used to separate carotin from xanthophyll led to securing a crude carotin preparation which contained the fat-soluble vitamine in large amounts and a crude xanthophyll preparation which contained none or little of it.

EXPERIMENTAL.

For the demonstration of the presence of the fat-soluble vitamine in our extracts and extraction residues, rats were used as the experimental animals. The extracts fed in most cases—exceptions being noted in their proper places later—were evaporated on dextrin and as such incorporated in a basal ration of 18 per cent casein, 2 per cent agar, 3 to 5 per cent ether-extracted wheat embryo and 4 to 5 per cent salt mixture,[1] the remaining caloric requirements being furnished by dextrin. The animals were fed and watered daily and were weighed once a week. This has become the general routine procedure of the laboratory for practically all our experimental groups, some being weighed oftener, and has been found very satisfactory in that it offers excellent opportunity for the systematic observation of the changes in the condition of the animals with the progress of the experiment. The detailed notations which are the result of such observations have not been entered in the protocols as they are of no special interest to the reader. To us, however, they were indispensable as we could not always anticipate the final outcome of the experiments and therefore could not pass on the ultimate value of any sudden change in the animals' condition.

Solubility of the Fat-Soluble Vitamine of Carrots in Fats.

These experiments were carried out with lard and commercial corn oil, both of which contain no fat-soluble vitamine. 3,000 gm. of dried grated carrots were soaked with 500 gm. of the fat and allowed to stand 6 to 8 days at room temperature. They were then extracted with ether in the cold three successive times, most of the ether was removed *in vacuo*, and the final traces were dissipated by exposure to an air current. There remained 534 gm. of preparation in the case of the lard and 539 gm. in the case of the corn oil which represented extractives totaling 34 and 39 gm. respectively. Both preparations were of a deep orange color.

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[1] For composition of the salt mixture see Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 505.

The lard preparation gave little indication of fat-soluble vitamine having been extracted. When fed at a 10.8 per cent level equivalent to 60 per cent of dried carrots none of the four rats in the experimental group, although they were started at 52 to 74 gm., attained a weight greater than 116 gm. All of them developed xerophthalmia in one or both eyes after 10 to 12 weeks. It is probable that initially the preparation was not so deficient in the vitamine as the above citation of conditions would lead one to believe. We infer this from the fact that chemical changes were taking place in the preparation, for although the preparation was kept as much as possible in the dark and in the refrigerator bleaching progressed rapidly; in 5 months all the orange-yellow color had been lost. It will be recalled that in butter fat we have observed a destruction of the fat-soluble vitamine simultaneous with the destruction of the pigment by exposure to heat (5), a phenomenon which we have since observed under other conditions as well. As butter owes its color to carotin, the same pigment found in our lard preparation, the inference that the preparation may originally have contained some demonstrable fat-soluble vitamine is not to be considered an impossible one.

With corn oil, considerable evidence that the fat-soluble vitamine was extracted by it is presented in Chart 1. In Lot 640 on corn oil alone, all the rats died within a period of 10 weeks and Rats 2556, 2557, and 2558 developed undoubted cases of xerophthalmia. With corn oil extract of carrots, on the other hand, when fed at the same level equivalent to 60 per cent of carrots none of the rats (Lot 532) died until they had been 24 weeks on the experimental ration and only two, Rats 2130 and 2129, developed xerophthalmia which were abortive attacks. It is true that on this extract the amount of growth that took place was negligible, but the mere fact that the animals maintained their body weight over a period of 24 weeks and recovered rapidly and solvents made it appear highly probable that we were dealing with a lipoidal molecule. As the carotinoids are obtained by ether extraction of a saponified alcoholic extract of leafy materials we applied such a procedure in an attempt to secure an active preparation from alfalfa. In this we were entirely successful. Furthermore, a fractionation of this material by the methods used to separate carotin from xanthophyll led to securing a crude carotin preparation which contained the fat-soluble vitamine in large amounts and a crude xanthophyll preparation which contained none or little of it.

EXPERIMENTAL.

For the demonstration of the presence of the fat-soluble vitamine in our extracts and extraction residues, rats were used as the experimental animals. The extracts fed in most cases—exceptions being noted in their proper places later—were evaporated on dextrin and as such incorporated in a basal ration of 18 per cent casein, 2 per cent agar, 3 to 5 per cent ether-extracted wheat embryo and 4 to 5 per cent salt mixture,[1] the remaining caloric requirements being furnished by dextrin. The animals were fed and watered daily and were weighed once a week. This has become the general routine procedure of the laboratory for practically all our experimental groups, some being weighed oftener, and has been found very satisfactory in that it offers excellent opportunity for the systematic observation of the changes in the condition of the animals with the progress of the experiment. The detailed notations which are the result of such observations have not been entered in the protocols as they are of no special interest to the reader. To us, however, they were indispensable as we could not always anticipate the final outcome of the experiments and therefore could not pass on the ultimate value of any sudden change in the animals' condition.

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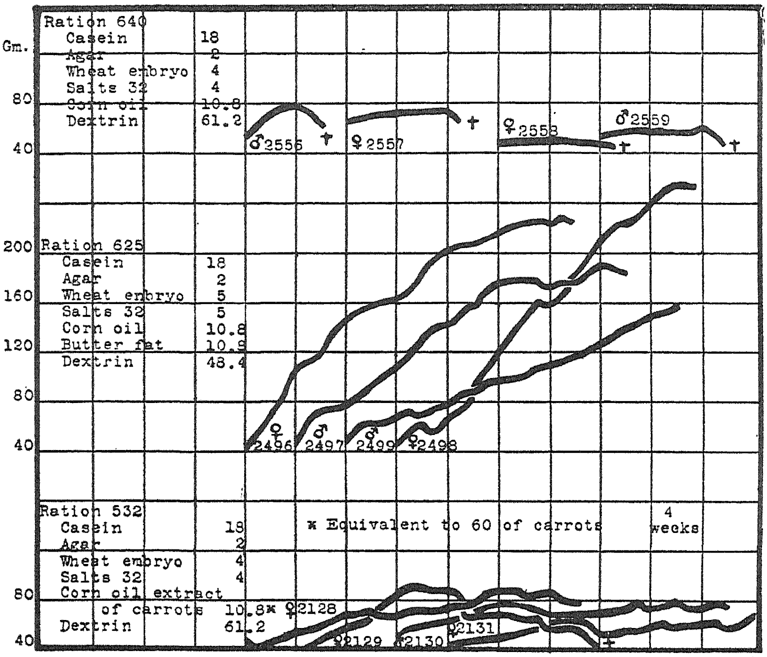

CHART 1.

Extractability of the Fat-Soluble Vitamine from Carrots by Fat Solvents.

In the preparation of these extracts 6 kilos of the grated carrots, first air-dried and then dried over calcium chloride, were treated with the respective solvents at room temperature in large percolators.

The extractions were continued for three 24 hour periods and were so conducted that the first extract was used on a second portion of carrots and that on a third, each percolator receiving at least one treatment with fresh solvent. Except in the case of the alcohol extract the solvents were recovered by vacuum distillation. With the alcohol extract, foaming on concentration proved so troublesome that it was found necessary to drive the alcohol off at room temperature with an air current. Ultimately all extracts were brought down on dextrin in an air current, the alcohol extract alone necessitating drying in a desiccator over calcium chloride as its high sugar content made it too hydroscopic for air drying.

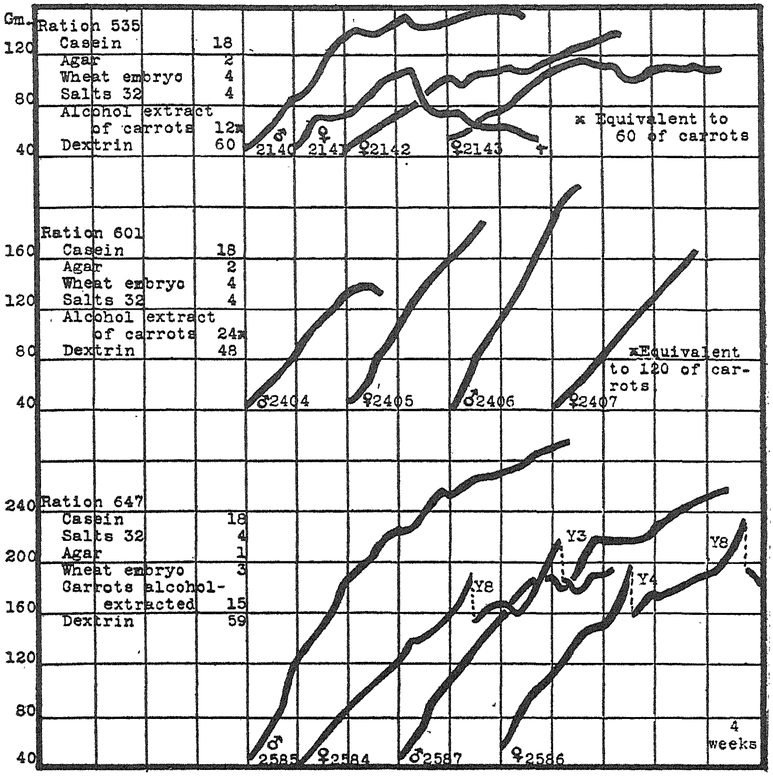

CHART 2.

TABLE I.

Preparation and amount of carrots or its equivalent fed.		Rat No.	Sex.	Weight of animals.						Notes on condition of animals.
				Initial.	4 wks.	8 wks.	12 wks.	16 wks.	20 wks.	
	per cent			*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
Ether extract of carrots	60	2124	♂	52	84	87	55	Dead.		Rats 2124 and 2126 developed xerophthalmia in both eyes before death.
		2125	♀	45	82	88	Dead.			
		2126	♀	42	92	119	82	Dead.		
		2127	♀	44	73	88	Dead.			
Carrots, ether-extracted	15	2588	♂	52	110	159	204	224	231	At end of 25th week condition of animals varied from poor to good. Rat 2589 died during parturition. Rat 2591 raised one litter of four young and one of two young.
		2589	♀	49	105	139	179	Dead.		
		2590	♂	57	114	179	242	272	268	
		2591	♀	57	110	163	179	180	212	
Benzene extract of carrots	60	2136	♂	70	106	143	156	158	155	All animals were in good condition.
		2137	♀	68	96	122	131	137	145	
		2138	♀	53	85	100	117	136	148	
		2139	♂	53	97	119	145	161	172	
Carrots, benzene-extracted	15	2596	♀	50	95	150	179	199	195	At end of the 25th week all animals were in good condition.
		2597	♂	59	135	197	236	267	294	
		2598	♂	54	119	160	231	267	292	
		2599	♂	55	105	172	219	240	248	
Chloroform extract of carrots	60	2192	♂	41	57	80	116	163	171	At end of the 22nd week animals in fairly good condition but growth not satisfactory. Rat 2194 had one eye suggestive of incipient xerophthalmia.
		2193	♀	37	51	56	80	115	150	
		2194	♀	43	53	77	113	150	171	
		2195	♂	40	63	87	113	130	150	

Carrots, chloroform-extracted......... 15	2592 2593 2594 2595	♂ ♀ ♂ ♂	57 50 62 60	114 92 110 98	162 132 139 135	216 207 194 176	250 165 225 200	265 221 225 203	When discontinued at end of 25th week condition of animals good. Rat 2593 raised one litter of six young beginning the 15th week.
Carbon disulfide extract of carrots... 15	2196 2197 2198 2199	♀ ♀ ♀ ♂	35 35 38 43	40 45 62 72	55 54 82 101	65 58 93 123	72 46 115 148	80 Dead. 125 160	10th week Rat 2196 had xerophthalmia in both eyes; later they improved. Rat 2199 had xerophthalmia in one eye in 11th week, in 13th week eyes were normal. Growth poor.

All the extracts were fed at a level of intake equivalent to 60 per cent of carrots, and in a few cases equivalent to 120 per cent as well. In addition some of the extracted carrots were incorporated in an otherwise fat-soluble vitamine-free ration to determine the extent of their extraction.

In Chart 2, Lots 535, 601, and 647 represent graphically the results obtained with the alcohol extract and the extracted residue. As seen by the growth curves not until the extract was fed at such a level that the equivalent of 120 per cent of carrots was consumed was growth satisfactory. This indicates a very poor solubility of the vitamine in alcohol as carrots themselves fed at a 15 per cent level completely cover the animals' requirements. Further evidence that the carrots had not yielded any significant amounts of the vitamine to the alcohol is seen by the performance of the rats on the extracted carrots in Lot 647. They grew at an entirely normal rate and Rat 2586 even raised ten out of twelve young from two litters and maintained herself in excellent condition.

In Table I are presented data of the growth of rats on rations restricted in their fat-soluble vitamine content to that introduced with ether, benzene, chloroform, and carbon disulfide extracts of carrots, and the carrot residues. All the extracts were fed at such a level that an amount equivalent to 60 per cent of carrots was introduced in the ration. The extracted carrots were fed at a 15 per cent level.

By comparison of the weights of the animals receiving the extracts with those receiving the residues it is seen that none of the extracts was entirely satisfactory, ether giving the poorest results and chloroform and benzene the best. It appears significant that, while all the extracts were originally highly colored, the ether extract bleached very rapidly and after a few months, though kept in the dark and in the refrigerator when not in use, it had lost all color. The other extracts retained their color fairly well though a year later it was observed that the carbon disulfide preparation when set aside in diffuse light gradually lost its color in the course of a few weeks. Unfortunately the other extracts had all been used up and were consequently no longer available for this comparison, but it appears rather suggestive of the chemical nature of the fat-soluble vitamine that here again with the destruction of the pigments there occurs a simultaneous disappearance of the vitamine. As in the case of the residue from the alcohol extraction, the ether, the benzene, and the chloroform extraction residues all gave little evidence of having lost large amounts of their vitamine. The carbon disulfide extraction residue was not fed.

The Extractability of Fat-Soluble Vitamine from Alfalfa.

With alfalfa, as with carrots, sufficient information on the fat-soluble vitamine content was available so that extractions could be carried out with a fair degree of assurance that although measurable quantities of vitamine were present the amounts were not so large that differences in content would not be readily perceptible. However, as a precautionary measure, to insure that a sufficiency of vitamine was present some of the material used was fed at a 5 per cent level in a fat-soluble, vitamine-free, but otherwise sufficient, ration. The alfalfa was a western grown sample available on the market as a meal for stock feeding purposes.

After its vitamine content was established, extractions were made with water, with ether, with benzene, and with alcohol, all extractions being conducted in the cold.

In the water extraction 1 kilo of alfalfa was soaked in a percolator with 6 liters of distilled water and gradually allowed to drain. As the water drained off more was added so that in the course of 2 days 8 to 10 liters of extract were accumulated. The alfalfa was then removed and dried before an electric fan. The extract was brought down on dextrin by the same means. The ether extract and the alcohol extract were made in a similar way except that in the case of the alcohol most of the solvent was distilled off in a water bath and recovered. This portion was also ultimately dried *in vacuo* as it proved to be very sticky. All extractions were continued for 48 hours with frequent renewal of solvent. In the preparation of the benzene extract 1,200 gm. of alfalfa were extracted with the solvent in three percolators, the extract of the one being used for the extraction of the second and that again for the third, a total of 7 kilos of solvent being used. At the end of the extraction most of the solvent was removed from the alfalfa by a powerful plant press and the remainder volatil-

TABLE II.

Preparation and amount of alfalfa or its equivalent fed.		Rat No.	Sex.	Weight of animals.						Notes on condition of animals.
				Initial.	4 wks.	8 wks.	12 wks.	16 wks.	20 wks.	
	per cent			*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
Alfalfa, unextracted..	5	2680	♀	49	95	140	185	180	175	Rat 2681 was rather thin. Other animals were in good condition.
		2681	♂	47	102	177	215	215	230	
		2682	♂	62	148	192	210	229	253	
		2683	♀	65	100	135	172	179	180	
Alfalfa, water-extracted...........	5	2552	♂	45	99	135	155	170	180	No xerophthalmia. Animals in poor to fair condition.
		2553	♀	42	92	130	149	157	170	
		2554	♂	43	97	138	150	200	225	
		2555	♀	61	95	136	134	160	167	
Water extract of alfalfa..............	20	2856	♂	69	165	120	Dead.			Rats 2856 and 2857 developed xerophthalmia, the others did not.
		2857	♀	56	122	Dead.				
		2858	♂	54	155	"				
		2859	♂	45	127	95	Dead.			
Alfalfa, ether-extracted..........	5	2568	♀	54	74	107	120	162	158	Rats 2568 and 2571 looked rough; malnutrition of skin. Rat 2570 was blinded by xerophthalmia.
		2569	♂	64	93	165	185	137	Dead.	
		2570	♀	58	78	104	141	149	100	
		2571	♂	60	120	174	192	242	232	
Ether extract of alfalfa............	20	2548	♂	45	92	152	205	245		Condition of animals good except Rat 2549, which was very wild and nervous when discarded.
		2549	♀	40	57	67	81	90		
		2550	♀	45	89	120	145	169		
		2551	♂	52	82	122	139	132		

Alfalfa, benzene-extracted...........	5	2692	♂	64	147	195	225	197		All the rats suffered more or less from cutaneous malnutrition especially evident on the ears, nose, tail, and feet.
		2693	♀	45	87	117	93	Dead.		
		2694	♂	52	69	90	111	119		
		2695	♀	48	62	72	101	98		
Benzene extract of alfalfa............	5	2868	♂	55	142	184	199	200		All in good condition when discontinued. Rat 2871 raised two out of a litter of three young.
		2869	♀	62	109	135	180	210		
		2870	♂	75	140	168	197	185		
		2871	♀	52	102	142	147	155		
Alfalfa, alcohol-extracted...........	5	2684	♂	50	85	Dead.				While none of the rats developed xerophthalmia, the eyes of all were either small or slightly swollen and red before death; suggested incipient attacks of xerophthalmia.
		2685	♀	52	115	"				
		2686	♂	64	110	77	Dead.			
		2687	♀	47	45	50	"			
Alcohol extract of alfalfa............	20	2948	♂	50	114	162	195	238	252	All in good condition.
		2949	♂	47	109	172	207	253	290	
		2950	♀	38	80	107	125	152	175	
		2951	♂	57	97	106	105	123	150	

ized at room temperature in an air current. The extract was brought down to a small volume *in vacuo*—after the addition of 5 gm. of calcium carbonate to neutralize the acidity—and then evaporated at room temperature on dextrin.

The results of feeding these extracts and their residues are brought out in Table II. The residues were fed at a 5 per cent level—as thereby any appreciable removal of vitamine would be revealed—and the extracts were fed at such a level that an intake equivalent to either 5 or 20 per cent of alfalfa in the ration was obtained.

Water, it will be noted, proved to be a very poor solvent for the vitamine as the rats dependent for their fat-soluble vitamine on a water extract of alfalfa died before 12 weeks of the experimental period had elapsed; moreover on the extracted residue they grew at a rate but slightly inferior to that observed on unextracted alfalfa. The slight discrepancy was no doubt occasioned by a partial destruction of the vitamine in the extractions as the early development of two cases of xerophthalmia in the group of animals on the extract makes it appear very improbable that it contained the vitamine.

Ether, U. S. P., freed from most of its water and alcohol over calcium chloride proved far more efficient than water in effecting a removal of the vitamine. On the alfalfa residue the rats were, however, still able to grow for a considerable time, but by the 20th week one rat had died and one had developed xerophthalmia. The extract fed at a level equivalent to 20 per cent of alfalfa proved fairly satisfactory.

Benzene gives indications of being a good solvent in view of the fact that the rats were able to grow well and even rear a few young on the extract fed at a level equivalent to 5 per cent of alfalfa. The residue also supported growth for some time, but when the experiment was discontinued the animals by the poor nutritive state of their skins and their decline in body weight gave evidence of an insufficiency of vitamine in their intake.

Alcohol, even when used in the cold, and without special precautions such as were used with benzene, appears to be an excellent solvent. This statement must be qualified as the extract was fed at a high level and therefore we have no assurance that smaller amounts would have been equally efficacious. This would naturally follow if we were certain that no destruction of the vitamine had occurred in the process of extraction as the extraction residues were found so inactive. We are inclined to believe that the alcohol extract contained a large excess of the vitamine and therefore that very little destruction had occurred, as a similar extraction carried out with hot alcohol when distinctive processes would be accelerated gave us responses in growth of entirely equal magnitude.

Extractability of Fat-Soluble Vitamine From Maize.

Our observations on maize are limited to extraction with ether and alcohol. The data are to be considered as merely supplementary to those obtained with alfalfa, for while yellow maize contains considerable quantities of the fat-soluble vitamine it is questionable that we will ever use it for extensive preparations, as at the most its content of vitamine is only about one-seventeenth of that of alfalfa. In this work we used part of the same lot of Golden Glow maize of which the vitamine content was already known (4).

The ether extraction was carried out in a large Soxhlet extractor with U.S.P. ether freed from alcohol and water by calcium chloride. For the first 4 weeks the growth of the animals on the ether-extracted residue was so phenomenal, Chart 3, Lot 635, that it was decided to increase the extraction time—which had been 6 hours—to 18 hours. Even when fed on this more highly extracted maize, the rats continued to grow rapidly and maintained an excellent appearance. The feeding trials gave no evidence that any of the fat-soluble vitamine had been removed. The ether extract was not fed.

Our results with alcohol were of an entirely different order as alcohol proved to be an excellent solvent. At first we extracted the maize with cold alcohol using the completeness of removal of yellow pigment as the criterion for the progress of extraction of the fat-soluble vitamine. Cold alcohol extraction proved too time consuming so that later extractions were made at first with hot alcohol on the coarsely ground material in large Soxhlets and then followed by a cold alcohol extraction on the maize reground to a fine meal. It had been found that the hot alcohol extraction could not be carried out on the finely ground maize as it invariably packed in the extractor to an impermeable mass. By the above mentioned course of procedure completeness of extraction was readily obtained, but we were not certain but that some destruction of the vitamine might have been brought about by the long continued boiling of the alcohol extracts. To minimize this, the hot alcohol extraction was interrupted at 6 hour intervals to enable the alcoholic solutions to be replaced with fresh alcohol. Three 6 hour periods were found sufficient for the extraction. The combined hot and cold extracts were

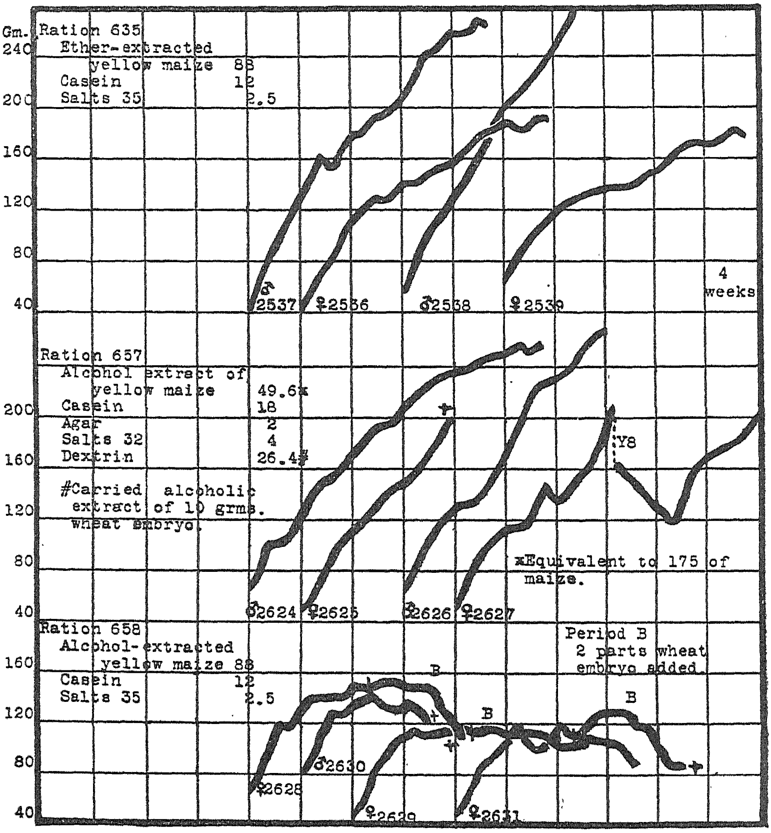

CHART 3.

concentrated on the water bath, with recovery of the solvent, and finally were evaporated on dextrin for introduction into the rations.

The maize residues from the first cold alcohol extractions were fed to Lot 658, Chart 3. They were practically free from pigment, but here and there traces of yellow pigment were still discernable in some of the larger flint particles. As seen in the chart, even when these residues were suitably supplemented with protein and salts they did not cover the requirements of the animals as their growth was of but short duration. It was a bare possibility that the failure of growth to take place was partly due to a deficiency of water-soluble vitamine in the ration though earlier experiments of the laboratory had shown that the water-soluble vitamine is extracted from maize by alcohol with great difficulty. To eliminate this criticism the ration after 10 weeks was further supplemented with ether-extracted wheat embryo and as noted in the chart no improvement in the performance of the animals resulted. Rats 2628, 2629, and 2631 later developed xerophthalmia so that no reasonable doubt as to the deficiency of the ration in its fat-soluble vitamine content can be entertained.

In correlation with the above, and furthermore as evidence of the stability of the fat-soluble vitamine in the alcoholic extract, we have the entirely satisfactory growth of the animals in Lot 657, Chart 3. These rats received the alcoholic extract of maize equivalent to 175 per cent of the grain and therefore but little more than twice the amount of vitamine required by the animals as measured by the original content of maize equivalent. The excellent growth performance of the animals, together with the fact that Rat 2627 even raised two out of her litter of eight young, bears testimony to the fact that the fat-soluble vitamine is readily extracted from maize by alcohol and, furthermore, that it is fairly stable under these conditions.

Fractionation of Fat-Soluble Vitamine Extracts.

The extractability of the fat-soluble vitamine having been demonstrated, we next attempted to formulate a procedure which might give promise of separating the vitamine from contaminating substances in the extracts. From its occurrence in nature, it appeared plausible that methods designed for the isolation of pigments of the carotinoid type, such as carotin and xanthophyll, might possibly give a preparation rich in this dietary essential.

From the sum total of our experience on the extractability of the vitamine, benzene and alcohol appeared to be the most promising solvents and of these alcohol was selected because it is usually obtainable free from undesirable impurities and furthermore is readily removable from the extractives. As is well known, one of the methods employed in the separation of the carotinoids is the saponification of the pigment extracts which leaves them, together with other non-saponifiable substances, intact. Later these are then extracted from the saponified mixture with ether and subsequently crystallized from large volumes of solvent to the desired degree of purity. On these general experimental premises a number of preparations were made, but it has not yet been considered advisable to attempt fractionations by crystallization at least until more data on the stability of the fat-soluble vitamine are available.

In the first preparation the procedure was as follows: 3 kilos of alfalfa meal were extracted with cold alcohol in large percolators in the course of a few days. In these extractions the alfalfa was soaked with the alcohol and enough additional solvent poured on so that the material was well covered. After 24 hours extraction the percolator was drained, again filled up with solvent, extracted for 24 hours, and again drained. This was repeated once more and then, after washing the residue with a little additional alcohol, all extracts were united and distilled in a water bath down to a volume of about 2 liters. The concentrated extract obtained was of a dark green color, due to the large amount of chlorophyll extracted. For the saponification 225 cc. of 20 per cent alcoholic potassium were added and allowed to stand over night. This failed to complete the saponification so that it was found necessary to add 50 cc. and later another 25 cc. of the potash solution. This completed the saponification and the formation of the ether-insoluble potassium chlorophyllin in an additional 6 hours. The mixture was extracted repeatedly with ether till practically all yellow pigments had been removed, the ether solution then washed with water to remove alkali and salts, and this in turn washed with ether. All ether extracts were then united, evaporated on the water bath to a small volume, and then again washed with a small amount of water. The solution thus obtained was of an orange-red color slightly tinged with brown but free from all green by reflected light. It was brought down on dextrin before an air blast for incorporation in a basal fat-soluble vitamine-free ration. The results of feeding this extract are shown graphically by Lot 668 in Chart 4. When the experiment was discontinued after having gone 22 weeks all the rats were in a fairly good condition. The evidence was indisputable; the vitamine had resisted the treatment to which it was subjected and finally had been successfully concentrated in ether solution.

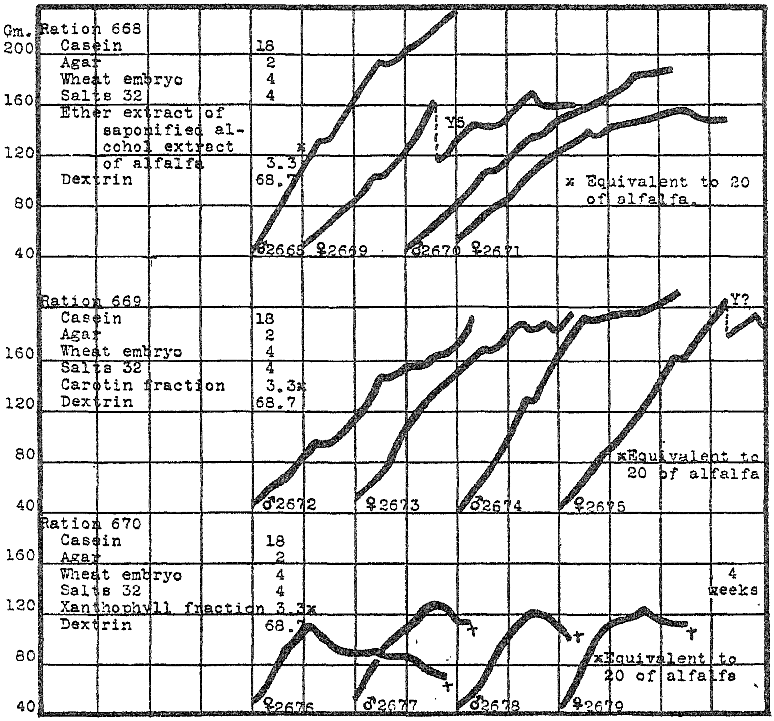

Chart 4.

Our next step was directed to determine whether the extract of the saponified alcohol extract of alfalfa could be successfully fractionated into an active and inactive fraction by a method such as has been used for the separation of the carotin and xanthophyll pigments. For this purpose 3 kilos of alfalfa meal were extracted as before with cold alcohol, the concentrated extracts were saponified with alcoholic potash, and then extracted with ether. Instead of evaporation on dextrin the ether extracts were freed from ether by exposure to an air blast. In the fractionation, the dark red residue was taken up in a mixture of alcohol and petroleum ether, transferred to a separatory funnel, and water added in small portions until the solution separated into two well defined layers—a lower one of diluted alcohol and an upper one of petroleum ether—both carrying considerable pigment. The respective layers were separated and reextracted—the alcohol fraction with petroleum ether and the petroleum ether with alcohol diluted with just enough water to bring about separation. Ultimately all alcohol solutions were united and all petroleum ether solutions were likewise united giving respectively the xanthophyll and carotin fractions. Each was evaporated on dextrin in an air blast and incorporated as the sole source of fat-soluble vitamine in an otherwise complete ration. The results from two such experimental groups are shown in Chart 4, Lots 669 and 670. They leave no doubt but what the carotin fraction contained an abundance of the vitamine while the xanthophyll fraction contained little or none of it. At the end of 17 weeks, when the feeding trials were discontinued, all the rats on the carotin preparation were in good condition, while all those on the xanthophyll fraction were dead, three having died before the end of the 10th week and one of these while suffering from a severe attack of xerophthalmia. The efficiency of this method of fractionation has been repeatedly demonstrated not only by different feeding trials, but also on different preparations. The presentation of these results together with detailed studies on stability, *etc.* will be reserved for later publications.

DISCUSSION.

In the light of the experimental data presented, there is abundant reason for assuming that attempts at isolation of the fat-soluble vitamine based on its solubility in fat solvents appear promising. As already stated, the difficulties of demonstrating its extractability were not so great as we had surmised when we were unaware of its stability under certain conditions and its lability under others. Furthermore we failed to anticipate the great variation with different substances in the ease with which the fat-soluble vitamine is yielded to certain fat solvents. It was no doubt the same premises that made McCollum and Simmonds (8) make the statement when speaking of the fat-soluble vitamine: "It is not extracted from plants with the fats by such solvents as ether, chloroform, benzene, or acetone, and is therefore not found in any fats or oils of plant origin. Hot alcohol does remove it from plant tissues." The reference to its solubility in alcohol is based on data obtained with the maize kernel which in themselves are not acceptable as McCollum, Simmonds, and Pitz (9) were of the opinion that maize generally was very deficient in the fat-soluble vitamine. Considering the evidence presented in a previous paper from this laboratory (4) such increased increments of growth as McCollum reported, due to the addition of an alcohol extract of maize to a basal maize ration, are entirely possible on the basal maize ration itself. Osborne and Mendel (10) also dissent from McCollum's point of view in regard to the non-extractability of the fat-soluble vitamine from leafy materials as they succeeded in obtaining a very potent oil from spinach and clover with U.S.P. ether. While our results with ether were not very successful we obtained abundant and unequivocal evidence that the fat-soluble vitamine can be extracted from plant materials with little loss of physiological potency with ether and with benzene, solvents which McCollum stated as being ineffective.

SUMMARY.

When carrots are saturated with lard or corn oil and then extracted with ether none or little of the fat-soluble vitamine is removed. The lard preparation gave no evidence of containing the vitamine, but the corn oil preparation contained it in small but persistent amounts.

Ether has little solvent properties for the fat-soluble vitamine as found in carrots; chloroform and carbon disulfide remove some of it; while alcohol and benzene remove considerable amounts of it. None is entirely satisfactory for extraction purposes.

Fat solvents applied to alfalfa gave far more satisfactory results. Even on an ether extract one rat increased in weight 200 gm. during an experimental period of 16 weeks. Benzene and especially alcohol were found good solvents. Water was entirely ineffective.

While the vitamine is not extracted from maize by ether, alcohol removes it quantitatively and with little, if any, destruction.

The fat-soluble vitamine as extracted from alfalfa with alcohol resists saponification with alcoholic potash in the cold. From such a solution on dilution with water it can be extracted with ether. On fractionation by differential solubility there can be obtained a petroleum ether-soluble portion also containing carotin and a dilute alcohol-soluble portion which contains xanthophyll; the former contains the fat-soluble vitamine in large amounts, the latter little or none of it.

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A STUDY ON THE SEPARATION OF THE PHYSIOLOGICALLY ACTIVE PORTION OF THE POSTERIOR LOBE OF THE PITUITARY BODY.

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(Received for publication, March 1, 1920.)

We are dealing in this paper with the uterus-contracting phase of the problem only. The basic material employed was a desiccated water extract, made by macerating and boiling finely minced posterior lobes with sufficient distilled water containing 0.25 per cent of glacial acetic acid to make the final volume represent 10 per cent of the fresh gland. On desiccation the liquid yielded about 0.7 per cent of total solids in the form of light yellowish hygroscopic scales. Several batches were thus made and combined into a uniform sample which represented the glands from 5,000 cattle. The chemical analysis of this extract did not reveal any striking peculiarities. The ash was mostly water-soluble, of alkaline reaction, and yielded CO_2 on addition of acid.

Ether, chloroform, petroleum ether, and methyl alcohol were ruled out as solvents for the active principle since the first three did not yield therapeutically active preparations and methyl alcohol extracted more impurities and less of the active principle than did ethyl alcohol. It was definitely established that the physiologically active portion was practically insoluble in absolute ethyl alcohol, very sparingly soluble in cold 95 per cent alcohol, but quite freely extracted with boiling 95 per cent alcohol.

10 gm. portions of the dried water extract, powdered over a 100 mesh sieve were extracted ten times with 50 cc. portions of 95 per cent ethyl alcohol at 20°C., and then eight times with equal volumes of boiling alcohol.

The combined cold alcohol extracts were evaporated until crystals appeared. After complete crystallization they were filtered, washed with alcohol and ether, and found to constitute 3 per cent of the dried basic water extract. On recrystallization from water and analysis they were found to consist of approximately 67 per cent potassium chloride and 33 per cent sodium chloride. If the crystals are not separated at this stage they will appear in the dried residue and are then removed with difficulty. During the earliest experimentation where only small amounts of extract were available, these crystals escaped detection until the alcoholic extracts were evaporated to paste consistency. They could then readily be observed with the naked eye. The microscope revealed beautiful large cubes and fern leaf formations embedded in the yellow paste. The remaining alcohol extract, after removal from the crystals, was evaporated to dryness, a small portion at 50°C. and the balance at about 70°C. The total yield was 23.4 per cent of the dried basic material. The extracts were yellowish brown in color, exceedingly hygroscopic, and possessed a characteristic pungent odor. Analysis showed this fraction to be rich in water-soluble mineral matter and organic extractives of a non-protein nature.

The hot alcohol extracts filtered clear when boiling hot, but yielded a white amorphous fraction on the slightest cooling, while the active substance remained in solution throughout. If the active principle were sparingly soluble in cold and freely soluble in hot alcohol, it would naturally be precipitated with the inert material on cooling, but such is not the case. We are apparently here confronted with some form of hydrolysis whereby a protein complex is split by alcohol. The amorphous precipitate amounted to only 1.2 per cent of the dried basic material and was too small for quantitative analysis. It was of a protein nature, soluble in water, and physiologically inert. The filtrate from this amorphous fraction was evaporated to dryness, one part below 50°C., and another at about 70°C. The total yield was 14.6 per cent of the dried basic material. This preparation was yellowish in color, very hygroscopic, and readily soluble in water. It contained a large amount of organic and inorganic salts and the total nitrogen was higher than that of the cold alcohol fraction, but not equal to that of the basic water extract.

The alcohol-insoluble residue was dried, and averaged 57.8 per cent of the dried basic material. It formed a cream-colored powder, much less hygroscopic than the alcohol extracts, and only partly soluble in water. The ash consisted principally of water-insoluble phosphates. The total sum of moisture, ash, and N × 6.25 approaches 100 per cent, indicating that this fraction is principally protein.

The physiological activity of all preparations was determined by the isolated uterine strip method. Samples of the original water extract before desiccation were ampuled, sterilized, and used as control. It will be seen from Table I that while the activity of the water extract was not affected by desiccation at 50–70°C. that of the alcohol extracts was markedly changed. The hot alcohol extract, which was dried below 50°C., was about 3.5 times stronger than the control, while the one dried at 70°C. was only 1.5 times as strong, plainly indicating the extraordinary sensitiveness of the active substance in this form to heat during drying. An independent experiment brought to light the fact that prolonged boiling of the dried water extract with alcohol, under reflux condenser, and evaporation of the extract at 100°C. rendered the preparation inert. We also found that while the hot alcohol extracts were exceedingly sensitive to heat on drying they retained their activity quite well in water solution and could be autoclaved without material loss in physiological value. The cold alcohol extract was only one-seventh and the alcohol-insoluble residue about one-fifth the strength of the control. The distribution of the physiological activity in the various fractions is as follows:

	per cent
Cold alcohol extract	3.5
Hot " "	51.0
Alcohol-extracted residue	11.5
Loss in process (by difference)	34.0

Most, if not all, of the loss in physiological activity in our present method of separating the active portion evidently occurred during the desiccation process of the hot alcohol fraction.

Table I shows that the hot alcohol extract, the most active physiologically, responded only slightly to the biuret test and to protein and alkaloidal precipitants. Experiments were carried out with uranium acetate, phosphotungstic acid, and tannic acid

TABLE I.

	Original 10 per cent acidulated water extract of the fresh posterior lobe.	Same extract desiccated.	Cold ethyl alcohol extract.	Crystals formed during evaporation of cold ethyl alcohol extract.	Hot ethyl alcohol extract.	Precipitate formed during cooling of hot alcohol extract.	Alcohol-extracted residue.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Yield................		100.0	23.4	3.0	14.6	1.2	57.8
Moisture.............	99.3	6.95	1.75		3.32		3.65
Total nitrogen........		11.16	7.25		9.87		13.94
Ash..................		13.87	21.30	100.0	16.38		7.14
Cl...................		1.93	1.73	51.95	1.75		Negative.
P....................		1.00	0.50	Negative.	0.70		1.44
K....................		6.56	Present.	34.79	Present.		Negative.
Na...................		0.93	"	13.26	"		"
Reaction to litmus of water extract of ash.		Alkaline.	Alkaline.	Neutral.	Alkaline.		Neutral.
CO_2 in ash............		Present.	Present.	Negative.	Present.		Negative.
Physiological activity compared with original water extract; desiccation at 50°C.	Control.	Equal to control.	15 per cent of control.	Inert.	3.5 times stronger than control.	Inert.	20 per cent of control.
Physiological activity; desiccation at 70°C.	"	" "	" "	"	1.5 times stronger than control.	"	" "

Biuret reaction........	Strong.	Strong.	Doubtful.		Positive.	Very strong.	Strong.
Tannic acid..........	Heavy precipitate.	Heavy precipitate.	Slight precipitate.		Precipitate.	Heavy precipitate.	Heavy precipitate.
Phosphotungstic acid..	" "	" "	" "		"	" "	" "
Potassium mercuric iodide.............	Precipitate.	Precipitate.	" "		Slight precipitate.	Precipitate.	" "
Picric acid...........	"	"	Turbidity.		Heavy cloud.	"	" "
Bromine water........	"	"	"		Slight precipitate.	"	" "

on freshly prepared acidulated water extracts of the gland. The resulting filtrates and precipitates were freed from respective reagents in the appropriate manner. In each case the preparations from both filtrate and precipitate possessed sufficient uterus-contracting power to render this method of separating the physiologically active portion of the gland incomplete. This condition further indicates split products. When picric acid in alcohol was added to a water extract of the fresh gland a precipitate, partly flocculent and partly crystalline, was formed on standing. The crystals were found to be identical with the inert salts described above.

SUMMARY.

Evidence is furnished which indicates that the uterus-contracting active principle of the posterior lobe of the pituitary body does not occur in the fresh gland in free or crystalline form, but is linked to or part of some protein complex.

In its original protein association the uterine stimulus is insoluble in ether, petroleum ether, chloroform, and practically insoluble in absolute alcohol. It is sparingly soluble in 95 per cent alcohol, but yields a highly active split product when treated with hot 95 per cent alcohol. The split product is amorphous in nature, very hygroscopic, and much more sensitive to desiccation than the original basic material from which it was derived.

The crystalline bodies naturally occurring in and isolated from the posterior lobe are inorganic salts possessing no uterus-contracting power.

Alkaloidal reagents only incompletely precipitate the active fraction of the water extract of the gland.

The work is being continued.

EXPERIMENTS ON THE UTILIZATION OF SALEP MANNAN.

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(Received for publication, March 23, 1920.)

In 1911 the author[1] reviewed the literature on the utilization of various polysaccharides from plant sources and reported some experiments on the utilization of mannan derived from the tubers of *Orchis* and *Eulophia*, popularly known as salep. It was then shown that this anhydride of mannose, common in the dietary of certain peoples in eastern Europe, was not hydrolyzable by enzymes of saliva, malt diastase, and pancreatic and intestinal juice, but that in feeding experiments it disappeared almost completely from the alimentary tract in man, the coefficients of digestibility in four experiments on two subjects being in three cases 100 per cent and in the fourth 94 per cent. Digestion of salep mannan with fecal bacteria resulted in the production of appreciable amounts of sugar, as has since been shown by Okey [2] to be the case with inulin.

There has never been any convincing evidence that carbohydrates resistant to the normal action of body enzymes can play a significant rôle in human nutrition, though attempts have been made to demonstrate a rise in the respiratory quotient after their administration, both in the case of inulin [3] and of agar-agar.[4] The respiratory quotient after administration of 200 gm. of inulin reached a maximum of only 0.89 in the 3rd hour after ingestion as compared with a rise to 0.92 in 2½ hours after feeding 150 gm. of oatmeal; and the quotient for soluble agar (100 to 110 gm.) reached a maximum of 0.86 in the 4th hour, the values in the preceding and succeeding hours being little if any above the fasting level. It does not seem therefore that sugar produced by fecal bacteria from such carbohydrates as inulin is available in any considerable amount for the host. Nevertheless, it appeared worth while to test this point in regard to salep mannan as it had been tested for inulin. The experiments herein reported were carried out before 1915, when they were interrupted because the supply of salep was cut off by the war.

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[1] Swartz, M. D., *Trans. Connecticut Acad. Arts and Sc.*, 1911, xvi, 247.

[2] Okey, R., *J. Biol. Chem.*, 1919, xxxix, 149.

[3] Goudberg, A., *Z. exp. Path. u. Therap.*, 1913, xiii, 310.

[4] Lohrisch, H., *Z. exp. Path. u. Therap.*, 1908–09, v, 478.

EXPERIMENTAL.

Coefficient of Digestibility.

In the course of this work determinations of the coefficient of digestibility were made on several more subjects, with larger quantities of material than had been hitherto used. Two healthy young women, each weighing 70 kilos, consumed in 3 days a total of 75 gm. of salep mannan as a part of a simple cellulose-free ration. The experimental period was preceded and followed by 4 day periods in which the same diet was used, save that tapioca took the place of salep. The mannan was extracted from the tubers with water; the starch present digested off; the mannan precipitated by alcohol, so as to remove the sugar from the starch digestion; dissolved and reprecipitated; washed with ether, and dried. It was then in the form of a convenient white powder, dissolving readily in water. On account of its gelatinizing quality, the ingestion of any considerable amount was difficult, and led to the use of the dry powder in capsules in subsequent human feeding experiments. The glucose equivalent of the salep eaten was 61 gm., and the total excess of carbohydrate in feces during the salep period as compared with the fore and after periods was 1.7 gm. (Subject M) and 2.8 gm. (Subject H) reckoned as glucose, giving coefficients of digestibility for the mannan of 97 per cent and 95 per cent respectively (Table I).

TABLE I.

Determination of Coefficients of Digestibility.

	Subject M.	Subject H.
	gm.	*gm.*
Total carbohydrate intake per day	315	315
Carbohydrate in feces per day, fore period	0.53	0.50
" " " " " after "	0.56	0.47
Average output per day, fore and after period	0.55	0.47
" " " " salep period	1.09	1.42
" excess, salep period over fore and after periods	0.55	0.94
Total excess, salep period (3 days)	1.65	2.81
Dextrose equivalent to salep eaten	61.15	61.15
Coefficient of digestibility for total carbohydrate, salep period, *per cent*	99.6	99.5
Coefficient of digestibility of salep mannan, *per cent*	97.3	95.4

TABLE II.

Summary of Coefficients of Digestibility of Salep Mannan in Human Feeding Experiments.

Subject.	Weight.	Length of salep period.	Salep fed per day.	Glucose equivalent of salep fed.	Coefficient of digestibility.
	kg.	*days*	*gm.*	*gm.*	*per cent*
Z (Woman)*	52	2	30†	22.7	100
X " *	42	2	30†	22.7	94
Z " *	52	2	20	18.0	100
P " *	56	2	20	18.0	100
M "	70	3	25	20.5	97
H "	70	3	25	20.5	95
R (Man)		1	45	20.5	97
B (Boy)	33	3	56	51.8	96
Average					97

* Swartz,[1] pp. 354, 355.

† Salep powder corresponding to 22.7 gm. of glucose.

In another case a diabetic man under supervision in a hospital was given in a single day 45 gm. of salep mannan in capsules, the diet otherwise being only broth, black coffee, and 2 ounces of whiskey. The feces were marked for the day by carmine and a dose of 1 ounce of magnesium sulfate was given with the morning broth. There was no inconvenience from gas formation as with inulin, and only 2.7 per cent of the carbohydrate fed was recovered in the feces, making the coefficient of digestibility in this case 97 per cent.

In a third case a 15 year old diabetic boy weighing 33 kilos served as subject in a series of experiments to determine the fate of salep in the diabetic organism. As a part of this work a 3 day digestion experiment was conducted, with a cellulose-free basal ration, to which was added salep mannan to the amount of 33, 65, and 70 gm. on 3 successive days. There was no discomfort, even from the 70 gm., and the coefficient of digestibility for the period was 96 per cent.

Altogether, the average of eight experiments on seven human subjects is 97 per cent, the range being from 94 to 100 per cent (Table II).

Influence on Nitrogen Output.

In the experiment on the two young women cited above, a careful study of the nitrogen balance was made. It was hoped in the beginning that any failure to utilize the salep might be detected by a change in the nitrogen output if the diets were planned just barely to meet the energy requirement, and a food known to be readily digested—tapioca—was used in the fore and after periods, but replaced by an equivalent amount of salep in the mid period. The amount of salep which the subjects found it possible to take was not sufficient, however, to exert any marked influence. Hence the most striking result of the study of the nitrogen balance was the increase in the fecal nitrogen during the salep

TABLE III.

	Period No.	Time.	Nitrogen in feces per day.	Weight of air-dry feces per day.
		days	*gm.*	*gm.*
Subject M.	1	4	0.64	14
	2	3	1.00	21
	3	4	0.65	15
Subject H.	1	4	0.59	13
	2	3	1.44	27
	3	4	0.64	14

period, amounting to 54 per cent in one case and 135 per cent in the other. There was also an increase in the volume of the dry feces, amounting to 46 per cent in one case and 105 per cent in the other. The amount of nitrogen and weight of air-dry feces per day are shown in Table III.

There was a slight fall in the urinary nitrogen in the salep period, but there were increases of 16 and 17 per cent respectively in the after period. This was probably due to a decided decrease in the urine volume during the salep period, and a subsequent "flushing out," the salep being very hygroscopic.

Fate in the Diabetic Organism.

Several attempts were made to discover whether salep could be converted into sugar in the diabetic organism. In the case of the diabetic man cited above, there was no sugar in the urine on the day of salep feeding, although the subject had been excreting sugar whenever carbohydrate was fed in ordinary forms. This same man on a previous day had received 10 gm. of salep without the appearance of sugar. The 15 year old diabetic boy was under observation for some months, the plan being to give salep in periods alternating with a salep-free but controlled diet, and to study the output of sugar, ammonia, and β-hydroxybutyric acid in the urine. The patient was not in a hospital and it was found toward the close of the work that he had not always followed instructions, even though his diet was administered in the laboratory. The most that can be said, therefore, with regard to sugar, was that there was no marked increase in any of the salep periods. In one of the best controlled periods lasting 5 days, salep was given averaging 56 gm. per day, but did not stop the production of β-hydroxybutyric acid which rose from 3.4 gm. on the day preceding the salep feeding to 15 gm. by the 2nd day, continuing about the same the 3rd day, and being promptly reduced by sodium bicarbonate administered in connection with green diet containing no salep on the 4th day. Both acetoacetic acid and ammonia showed slight increases during this period. While this work needs to be repeated if it is possible to get supplies of salep again, there was no evidence of salep being converted into sugar in the diabetic organism.

Glycogen Formation in Rabbits.

It was thought that in an herbivorous animal like the rabbit glycogen storage might possibly be demonstrated for this carbohydrate in the absence of specific body enzmes for its hydrolysis, through the activities of sugar-producing bacteria, as mannose has been shown to form glycogen readily.[5] Accordingly a number of experiments were conducted to test this point. The animals were fasted from 5 to 6 days and then fed salep mannan in solution by means of a stomach sound, for from 1 to 3 days. They were killed 12 to 15 hours after the last feeding and the livers immediately removed and analyzed for glycogen, according to Pflüger's method.[6] Control rabbits were fed by the same

TABLE IV.

Glycogen Storage in Rabbits.

Subject.	Initial weight.	Time fasted.	Time fed.	Material fed.	Amount fed per day.	Weight of liver.	Glycogen recovered.	Ratio of glycogen recovered to weight of liver.
	gm.	*days*	*days*		*gm.*	*gm.*	*mg.*	*per cent*
I (Control)	1,275	6				18	35	0.20
II "	1,522	5	1	Starch. Lactose.	10 5	32	209	0.65
III*	1,262	6	1	Salep.	30	28	35	0.13
IV	1,495	6	1	"	15	22	5	0.02
V†	1,700	5	3	"	12.5	29	22	0.08
VI‡	1,418	5	3	"	20	28	16	0.06

* The contents of the stomach and intestines were collected and hydrolyzed, and carbohydrate was determined as glucose. 7 gm. were obtained from the large intestine, 1.5 from the small, and 1.5 from the stomach, or a total of 10 gm. That this sugar was largely, if not wholly, mannose was shown from a rich yield of mannose hydrazones in the hydrolyzed solution.

† Stomach and intestines yielded 5.9 gm. of glucose.

‡ In this case the fermentation was very marked. The contents of stomach and intestines were removed and 4.8 gm. of the mannan isolated and identified.

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[5] Neuberg, C., and Mayer, P., *Z. physiol. Chem.*, 1902-03, xxxvii, 530.

[6] Pflüger, E. F. W., Das Glykogen und seine Beziehungen zur Zuckerkrankheit, Bonn, 1905.

method equivalent amounts of soluble starch alone, or of soluble starch and lactose together, the former proving preferable as the lactose induced diarrhea. The results of these experiments are summarized in Table IV. Two of the controls suffered accidents, but inasmuch as the storage of glycogen in the rabbit has been extensively studied, and the results with the salep are so definite as compared not only with the controls given in the table but with other work of this sort, it is felt that this loss was not serious. The rabbit fed only 15 gm. of the starch-lactose mixture showed a storage of 209 mg. of glycogen, while the storage in the salep rabbits resembles that of the fasted control, and in fact never reaches so high a percentage of the total weight of the liver. The four cases of salep administration gave practically starvation values. This is in sharp contrast to the results of feeding mannose as reported by Neuberg and Mayer [5] who found that from 8 to 10 gm. of mannose similarly administered yielded such values as 0.973, 0.606, and 1.01 gm. of glycogen. Salep seems to be even less capable of glycogen formation in the rabbit than is inulin.[7]

SUMMARY.

Salep mannan disappears almost completely from the human alimentary tract when fed in amounts varying from 20 to 56 gm. per day (corresponding to 18 to 52 gm. of glucose), as shown in eight feeding experiments in which the coefficients of digestibility range from 94 to 100 per cent, averaging 97 per cent. Four of these are reported in this paper.

The total nitrogen of the feces and the total volume of dry feces are increased by salep feeding.

Salep feeding did not result in glycosuria in one diabetic studied, nor did it arrest the production of β-hydroxybutyric acid in another.

Although rabbits readily store glycogen when mannose is fed, the administration of salep mannan resulted in no glycogen in rabbit livers above ordinary starvation values.

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[7] Nakaseko, R., *Am. J. Physiol.*, 1900–01, iv, 246.

Salep resembles inulin in the hydrolysis by fecal bacteria, and in the readiness with which it disappears from the alimentary tract, but it does not form gas so readily. There was no evidence of gas or diarrhea except in one rabbit. It is less capable of glycogen formation in the rabbit than is inulin.

AT WHAT LEVEL DO THE PROTEINS OF MILK BECOME EFFECTIVE SUPPLEMENTS TO THE PROTEINS OF A CEREAL GRAIN?*

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(Received for publication, March 27, 1920.)

Definite information on the quantity of milk proteins necessary to supplement with maximum efficiency for growth the proteins of a cereal grain is not available. The question is a decidedly practical one. Advice as to how much milk to be used daily per individual in the human dietary is now freely given—with recommendations varying from 1 pint to 1 quart—but on what scientific evidence such advice rests is not apparent. Experiments with growing swine on the proportion of skim milk necessary to supplement corn-meal (maize) have been conducted by Henry.[1] His results indicated that with one to three parts of skim milk to one part of corn-meal satisfactory growth could be made. These experiments were never longer than 10 weeks.

Manifestly the quantity of liquid whole milk to use as a source of vitamines or salts needed for normal growth will depend on the nature of the rest of the dietary. It is apparent, therefore, that a quantitative expression of the amount of milk needed daily may have little significance as far as vitamines and salts are concerned. This applies particularly to the mixed dietary of children. The situation may receive safer treatment if more importance is attached to the value of the proteins of milk and the basis for milk intake related to their supplementing power. Of course,

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* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Henry, W. A., *Rep. Wisconsin Exp. Station*, 1895, 7.

it is true that there may occur composite diets of plant origin, whose protein mixture may be of fair efficiency at low planes of intake; further, it is also possible for meat proteins to supplement effectively the plant proteins, thereby lessening the quantity of milk needed where meat is part of the diet; but as far as we are aware there is no mixture of proteins—with the possible exception of egg proteins—when used either alone or as a supplement to the cereal grain proteins—that is equal in efficiency at as low a level as that of milk, which gives to the latter a unique place among foods.

There are two methods of experimental inquiry open for investigating this question. One is to measure over comparatively short periods of time the nitrogen retention of a growing animal on a protein mixture made from a cereal grain and milk; the other is to determine the rates of growth over longer periods of time of animals fed rations with varying proportions of cereal and milk proteins. We have used both methods; the first with growing swine and the latter with growing rats. The results of the experiments with the latter will be embodied in a later publication.

In our work on nitrogen retention with growing swine the method of procedure has already been described.[2] We chose to keep the cereal proteins used constant and allow the proportion of milk proteins to vary. With increasing levels of milk protein intake this procedure modified slightly the total plane of protein intake. In the several rations used the level of protein ingested varied from 8.6 to 10.9 per cent of the ration. The effect on the final conclusions would be to give slightly higher values of efficiency to the lower planes of protein intake. This affected in no way the interpretation of the data which can be seen in Chart 1. In this chart, for purposes of clarity, the corn intake is indicated as constant, while in the actual experiments, due to the variation in the size of animals, the amount of corn-meal fed varied. In Table I giving the detailed data the relative proportion of milk proteins to corn proteins is the same as shown in the chart. The therms supplied were calculated as *net* available energy.

The results secured are limited to corn-meal (maize) and milk, although they probably are applicable to other cereal grains; but in all probability they would not apply to a grain supplemented with a protein concentrate of plant or animal origin such as oil meal or meat. For example, 600 gm. of corn-meal supplemented with 100 gm. of oil meal may require less milk proteins for efficient nutrition than the level of proteins supplied by corn alone. This inference is made not only because oil meal supplements to some extent the proteins of corn-meal, but because of the higher level of proteins that would be supplied with the corn-meal-oil meal mixture. On the other hand it is known to us[3] that the corn proteins, if they could be fed at a high enough level, would meet the needs of a growing animal.

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[2] Hart, E. B., and Steenbock, H., *J. Biol. Chem.*, 1919, xxxviii, 267.

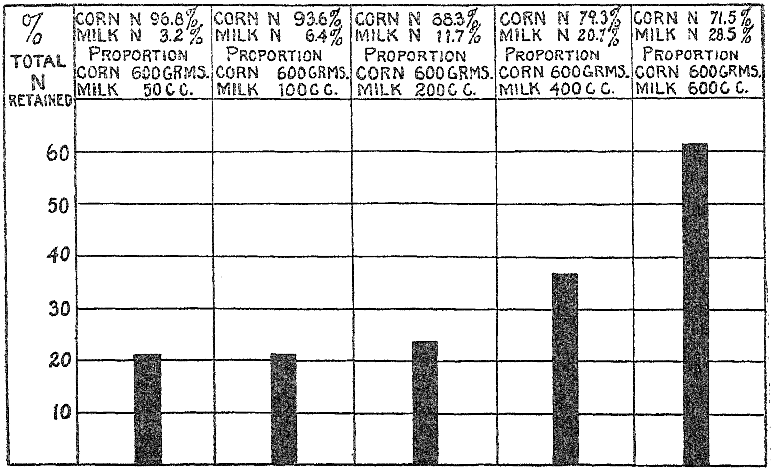

Chart 1. This chart shows the plane at which milk proteins must be used in order to supplement effectively the proteins of corn-meal.

Accepting Atwater's statement[4] of the daily food intake for growing children of 6 to 15 years of age at 443 gm. of dry matter (2,040 calories) composed of 75 gm. of protein, 43 gm. of fat, and 325 gm. of carbohydrates, the daily fluid intake of milk for an efficient protein mixture could be safely set at 1 pound (equivalent to 454 gm.). This would be liberal from the standpoint of an

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[3] Hart E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373. Johns, C. O., Finks, A. J., and Paul, M. S., *J. Biol. Chem.*, 1920, xli, 391.

[4] Atwater, W. O., quoted from Jordan, W. H., Principles of human nutrition, New York, 1917, 182.

TABLE I.

Complete Data on Nitrogen Balance.

Pig No.	Live weight.	Feed daily.	Therms provided daily.	Total N fed daily.	Total protein fed daily.	Protein in ration.	Total N fed 24 days.	N in feces 24 days.	N in urine 24 days.	Total N retained.	Absorbed N retained.
	lbs.			*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	44	600 gm. corn-meal. 50 cc. skim milk.	1.57	8.37	52.8	8.6	200.8	88.4	65.6	23.3	42
2	50	600 gm. corn-meal. 50 cc. skim milk.	1.57	8.37	52.8	8.6	200.8	100.9	47.7	25.9	52
3	47	600 gm. corn-meal. 50 cc. skim milk.	1.57	8.37	52.8	8.6	200.8	105.6	68.0	13.5	29
4	43	600 gm. corn-meal. 50 cc. skim milk.	1.57	8.37	52.8	8.6	200.8	81.8	72.3	23.3	39
Average										21.5	
5	49	700 gm. corn-meal. 117 cc. skim milk.	1.86	10.18	63.6	8.9	244.3	109.7	82.9	21.1	31
6	63	700 gm. corn-meal. 117 cc. skim milk.	1.86	10.18	63.8	8.9	244.3	133.8	80.0	12.5	28
7	51	700 gm. corn-meal. 117 cc. skim milk.	1.86	10.18	63.8	8.9	244.3	106.4	58.9	32.3	57
8	65	800 gm. corn-meal. 133 cc. skim milk.	2.12	11.61	72.6	8.9	278.6	142.2	74.9	22.1	45
Average										22 0	

9	87	800 gm. corn-meal. 266 cc. skim milk.	2.16	12.4	77.5	9.4	297.6	131.9	75.8	30.2	54
10	102	900 gm. corn-meal. 300 cc. skim milk.	2.43	14.0	87.5	9.4	336.2	142.6	107.9	25.6	44
11	104	900 gm. corn-meal. 300 cc. skim milk.	2.43	14.0	87.5	9.4	336.2	174.2	80.3	24.3	50
12	68	800 gm. corn-meal. 266 cc. skim milk.	2.16	12.4	77.5	9.4	297.6	116.7	115.6	21.9	37
Average										25.5	
13	75	800 gm. corn-meal. 533 cc. skim milk.	2.25	13.9	86.8	10.2	333.6	107.9	100.5	37.5	56
14	73	800 gm. corn-meal. 533 cc. skim milk.	2.25	13.9	86.8	10.2	333.6	106.0	99.7	38.4	56
15	80	800 gm. corn-meal. 533 cc. skim milk.	2.25	13.9	86.8	10.2	333.6	118.9	98.4	34.9	57
Average										36.9	
16	90	800 gm. corn-meal. 800 cc. skim milk.	2.33	15.4	96.1	10.9	369.6	57.4	85.4	61.3	72
17	85	800 gm. corn-meal. 800 cc. skim milk.	2.33	15.4	96.1	10.9	369.6	60.4	81.9	61.5	73
18	87	800 gm. corn-meal. 800 cc. skim milk.	2.33	15.4	96.1	10.9	369.6	65.4	80.8	57.7	73
Average										60.2	

efficient protein mixture, not only because of the high plane of protein intake required by the standard, but also because of the possibility that a mixture of proteins from diverse sources would, in themselves, have a fair efficiency. Basing the energy intake for children on the recent studies of Gephart,[5] then the milk consumption should be increased. In fact it would be double that suggested by the Atwater standard since these studies showed a daily intake of 4,000 to 5,000 calories by athletic boys.

From the results presented the inference might be made that normal rates of growth could not be secured until a highly efficient protein mixture was fed. This would appear to be contrary to what has been published in the literature. The apparently successful rearing by Hindhede[6] of his own children on low levels of protein of presumably only fair quality is a point in evidence. Probably the explanation of such results lies in the slower rates of growth of man, coupled with the possibility of increased consumption. Further, recent experiments by Osborne and Mendel,[7] where with rats and a level of 10 per cent of barley protein good growth was secured, make it more difficult to obtain a full and clear comprehension of this problem. In maintenance experiments with man, Sherman and Winters[8] did not obtain a positive nitrogen balance when practically all the protein of the diet came from corn-meal. A negative nitrogen balance was converted into a positive balance, when a certain amount of milk supplemented the corn-meal.

However, from the standpoint of the greatest utilization of ingested protein it would seem that experiments should be directed toward finding the most efficient protein mixtures possible. It would be greater economy to use a mixture of barley protein and milk proteins at levels of effective supplementary relations rather than depend upon excessive intake of a protein mixture of low efficiency, and certainly not successful as the sole source of proteins with all animals, even of the same species.

The positive mathematical facts obtainable with cage experiments and nitrogen retention leave no doubt as to the relative merits of protein mixtures examined and dispel the constant doubt in growth experiments that increments in weight may not be protein storage alone, but in part may be due to an excessive storage of fat, water, *etc.* From the practical experiences of animal husbandmen in the growth of swine it has been demonstrated again and again that maximum rates of growth cannot be secured with the cereal grains fortified with all other nutritive factors and supplied unconsciously in forage, such as alfalfa pasture. A protein supplement of high efficiency such as tankage or skim milk has always been found necessary for maximum rates of growth. This experience harmonizes with our contention that it is always safer to use highly efficient protein mixtures in nutrition during growth. This by itself justifies the use of milk in the human dietary.

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[5] Gephart, F. C., *Boston Med. and Surg. J.*, 1917, clxxvi, 17.

[6] Hindhede, M., Protein and nutrition; an investigation, London, 1913.

[7] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 275.

[8] Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 301.

Our views are in agreement with the statement made by Sherman,[9] who has given careful consideration to the problem of the protein requirement of maintenance in man:

> "It is plainly desirable in all cases that grain products be supplemented by milk products, and it is clear that in providing for needs of growing children and of pregnant or nursing mothers the proportion of milk in the diet should be more liberal than it need be when only maintenance is concerned; this both because of the superior amino-acid make-up of the milk proteins and to provide amply for the mineral elements and vitamines as well."

SUMMARY.

The results show that a highly efficient protein mixture is not obtained until the proportion of liquid milk to corn-meal reaches 1:1; in this proportion the milk nitrogen will constitute approximately 30 per cent of the total nitrogen of the ration.

These data relate only to protein efficiency and of course are not to be interpreted as indicating the amount of milk necessary for providing an adequate supply of vitamines or salts to an animal with a growth rate similar to that of swine.

[9] Sherman, H. C., *J. Biol. Chem.*, 1920, xli, 97.

FERMENTATION CHARACTERISTICS OF CERTAIN PENTOSE-DESTROYING BACTERIA.*

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The pentoses, xylose and arabinose, have been termed nonfermentable sugars. In a general sense this is true, as the majority of microorganisms, yeasts and bacteria, cannot utilize the pentoses; however, there are certain forms which possess the ability to break down these sugars. Apparently these pentose-fermenting bacteria are widely distributed and no doubt play an important rôle in the economy of nature.

In previous publications[1,2] attention was called to the occurrence of certain pentose fermenters in soil, manure, sauerkraut, and silage. These organisms were isolated, their general properties described, and the name *Lactobacillus pentoaceticus, n.sp.*, was suggested. This group of the pentose fermenters is characterized chiefly by the rapidity with which it breaks down xylose and arabinose with the production of acetic and lactic acids, and traces of alcohol and carbon dioxide.

The present paper deals with the general fermentation characteristics of the pentose fermenters.

EXPERIMENTAL.

To determine the value of any substance as a source of carbon for an organism it is desirable to measure its growth in a synthetic medium free from any other source of carbon. In the case of the

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[1] Fred, E. B., Peterson, W. H., and Davenport, A., *J. Biol. Chem.*, 1919, xxxix, 347.

[2] Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, 1920, xli, 181.

pentose fermenters all attempts to grow these organisms in synthetic media failed. After several tests, it was found best to use yeast water medium for all fermentation tests. The yeast water alone contains a very small amount of available carbon compounds, hence a control, consisting of yeast water alone plus the proper organism, was run and in reporting results these controls have been subtracted. The yeast water used in this study of fermentation reactions was prepared as follows:

450 gm. of pressed yeast were steamed 3 to 4 hours with 4,500 cc. of tap water with occasional stirring. This infusion was then allowed to stand undisturbed until a heavy deposit of yeast cells and other suspended matter had settled to the bottom of the vessel. The portion above this deposit was carefully poured off and sterilized in liter flasks. These flasks of the sterile yeast infusion were allowed to stand for 1 to 2 weeks, when the supernatant liquid was syphoned off and used without clearing or filtering.

For a few of the experiments this medium was cleared with egg albumin and a clear yellow liquid resulted. The desired amount of the carbohydrate, 2 per cent unless otherwise stated, was then added; and if calcium carbonate was not to be used in the medium its reaction was adjusted to a hydrogen ion concentration of about pH = 7.0 by titration with phenol red. Accurately measured portions were then pipetted into Erlenmeyer flasks and sterilized at 12 pounds pressure for 30 minutes. The calcium carbonate was sterilized in powdered form and added to the culture medium immediately before inoculation. In many of the cultures, the indicator bromocresol purple was added and the acid produced neutralized by means of sterilized 1 N NaOH; unless otherwise stated, the sugars were sterilized in the yeast water.

The yeast water was found very satisfactory for the growth of the pentose fermenters. All the analyses were made in duplicate and frequently in triplicate, usually with closely agreeing results. Since no variation was noted in the products of fermentation from cultures kept under widely different tensions of oxygen, the organisms were cultivated in deep layers of culture solutions, usually 250 cc. of culture in a 300 cc. Erlenmeyer flask. Continued cultivation in the presence of certain carbohydrates apparently did not produce any change in the power of fermentation. All cultures were incubated at 28°C. for varying periods of time. The carbohydrates used were, with the exception of xylose,[3] Merck C.P. products. The substances studied were:

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[3] The xylose was obtained through the courtesy of Dr. C. S. Hudson, formerly Chief of the Carbohydrate Laboratory of the Department of Agriculture.

1. Pentoses: xylose and arabinose.
2. Methylpentose: rhamnose.
3. Hexoses: glucose, galactose, mannose, and fructose.
4. Disaccharides: sucrose, maltose, and lactose.
5. Trisaccharides: raffinose and melezitose.
6. Polysaccharides: starch, inulin, cellulose, and xylan.
7. Glucosides: salicin and esculin.
8. Alcohols: mannitol and glycerol.
9. Organic acids: pyruvic, lactic, succinic, malic, tartaric, and citric.

In all the work a 2 per cent solution of the various carbon compounds was used. Fermentation was indicated by a change in the hydrogen ion concentration and particularly by the amount of volatile and non-volatile acid produced. As a rule these organisms bring about a vigorous fermentation without noticeable gas accumulation in a fermentation tube. This is particularly true of the fermentation of the pentoses, xylose and arabinose. The volatile acid was determined by distilling with steam, and the non-volatile acid by extracting the residue from the steam distillation with ether in a Kutscher-Steudel extraction apparatus for 50 to 75 hours. In a number of cases the barium salts of the volatile acid and the zinc salts of the non-volatile acid were prepared and analyzed.

Additional studies of the chemical changes arising from the decomposition of these carbon compounds are now in progress. A report on this phase of the work will be given at a later date.

A Comparison of the Fermentation of Various Carbohydrates and Related Compounds.

Reaction.—Changes in the hydrogen ion concentration of culture media is frequently used as an index of fermentation. This method of study was employed with many of the common sugars and related compounds fermented in the absence of a neutralizing agent. The pH values were determined 14 days after the different media were inoculated with Cultures 41–11 and 118–8. The control tubes contained yeast water only and were inoculated with the same cultures. They gave a change of not more than pH 0.4. It will be seen from the data in Table I that all the carbohydrates used were fermented, except raffinose, with a decided change in the hydrogen ion concentration. The fermentation proceeds rapidly, especially in the case of xylose and fructose; and usually within 24 to 48 hours there is a well defined change in pH values. After the 4th or 5th day the true acidity of the xylose, glucose, galactose, and fructose cultures remains practically constant at about pH 3.6 to 4.0. No reversion from an acid reaction to a more alkaline was ever noted.

TABLE I.

Comparison of Acid Production from Various Sugars and Related Compounds.

No.	Carbon compound.	Culture 41-11.			Culture 118-8.		
		At beginning.	At end.	Difference.	At beginning.	At end.	Difference.
		pH	*pH*	*pH*	*pH*	*pH*	*pH*
1	Xylose	5.6	3.6	2.0	5.6	3.5	2.1
2	Glucose *a**	6.0	4.0	2.0	6.0	3.6	2.4
	Glucose *b*	6.8	3.9	2.9	6.8	3.8	3.0
3	Galactose	7.2	4.0	3.2	7.2	4.0	3.2
4	Fructose	6.4	3.9	2.5	6.4	3.9	2.5
5	Sucrose *a*	6.0	3.6	2.4	6.0	3.6	2.4
	Sucrose *b*	7.0	3.8	3.2	7.0	4.6	2.4
6	Lactose *a*	5.8	3.7	2.1	5.8	4.4	1.4
	Lactose *b*	6.8	3.9	2.9	6.8	Lost.	
7	Mannitol	6.6	4.3	2.3	6.6	4.3	2.3
8	Raffinose	7.0	6.6	0.4	7.0	6.4	0.6
9	Yeast water alone	7.0	6.6	0.4	7.0	6.6	0.4

* *a* and *b* denote different experiments.

The rate of acid formation is much slower in the case of sucrose, lactose, and mannitol than in the case of the other carbon compounds and the pH values are higher. The results indicate that the sugars, xylose, glucose, galactose, fructose, sucrose, and lactose, are readily decomposed, giving approximately the same pH limit of growth. The final reaction averages about pH 3.6 to 3.9 which may be termed the acid limit of fermentation for this group of organisms in this media. The mannitol is less easily decomposed and consequently gives a higher pH value, probably due to the slow rate of fermentation.

As has been noted by other investigators[4] it was observed that the greatest changes in hydrogen ion concentration often were accompanied by apparently little visible growth in the tubes. A vigorous fermentation with only a slight visible growth in the culture medium is especially noticeable in the case of xylose.

Undoubtedly changes in pH values furnish an easy means of indicating carbohydrate fermentation, but do not necessarily give a true picture of the utilization of these substances. For instance xylose in concentrations not in excess of 4 per cent is converted almost quantitatively into acetic and lactic acids; while glucose, galactose, and sucrose under the same conditions show only a small utilization of the added sugars.

Gas Production.—The method of studying carbohydrate fermentation by gas production in Smith fermentation tubes was tested. For this purpose 2 per cent yeast water solutions of xylose, glucose, galactose, fructose, sucrose, lactose, raffinose, and mannitol were used. The cultures were kept at 28°C. and examined daily for a period of 3 weeks. During this time no gas collected in the closed arm of any of the fermentation tubes.

Although gas production in a fermentation tube has been used as a common indication of carbohydrate decomposition, for the pentose fermenters this method is almost worthless since only traces of gas are produced. In the majority of cases where the fermentation tubes gave no visible gas production more accurate methods of analysis showed a strong fermentation with gas formation.

Keyes,[5] Clark,[6] and others have pointed out the inaccuracies of the fermentation tube for measuring gas production and the use of the results thus obtained as an index of growth. The decomposition by the pentose fermenters of glucose, galactose, mannose, and other sugars with the formation of CO_2, none of which can be collected in fermentation tube, offers striking examples of the fallacy of this method. In the case of these sugars, no gas is noted in the closed end of the fermentation tube and yet they ferment rapidly with the production of large amounts of CO_2 and organic acids. About 20 to 25 per cent of the glucose and galactose is converted into CO_2.

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[4] Ayers, S. H., Rupp, P., and Johnson, W. T., *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 782*, 1919, 21.

[5] Keyes, F. G., *J. Med. Research*, 1909, xxi, 69.

[6] Clark, W. M., *Science*, 1913, xxxviii, 669.

Comparison of Reaction and Gas Production.

Unfortunately measurements of the change in hydrogen ion concentration also fail to give a true idea of the fermentative value of the carbon compounds. Often the change in pH value may be well defined when only a small amount of the carbohydrate has been consumed. Although far superior to the fermentation tube test as a criterion of fermentation, the change in hydrogen ion concentration is open to certain objections. The change in pH values depends upon many factors, chiefly the amount and strength of the acid or acids formed. An organism may bring about complete utilization of the carbohydrate with the production of large amounts of alcohol, acetone, or other neutral bodies and the relatively weak carbonic acid. On the other hand the carbohydrate may suffer only a slight decomposition with the production of stronger acids, for example, acetic, lactic, and succinic acids, and consequently a well defined change in pH values.

In a study of the fermentation reactions, it is believed that the total acid as well as the kinds of acids should be considered. With this in mind attention was directed to the soluble acid products of fermentation.

In this connection it should be borne in mind that the chemical processes involved in the utilization of carbohydrates by bacteria are by no means simple; and undoubtedly the steps are much more complicated than was formerly supposed. The intermediate and often the end-products are difficult to demonstrate and are subject to changes depending upon the conditions of the fermentation. In this work only the products which accumulated during fermentation were measured. The carbohydrates are considered in the order of their availability and their chemical relation; the most easily fermented are given first.

Fermentation of Xylose, Arabinose, and Rhamnose.

The total amounts of volatile and fixed acids produced at different stages of growth from the pentoses, xylose, arabinose, and rhamnose, in cultures inoculated with 41–11 and 118–8 are tabulated in Table II. Xylose and arabinose are decomposed very rapidly by these organisms with the formation of large amounts of acid. In a previous paper[1] attention was called to the great rapidity and the completeness of the fermentation of xylose by *Lactobacillus pentoaceticus*.

TABLE II.

Fermentation of Pentoses.

	Culture No.	Time.	0.1 N acid in 100 cc. of culture.			
			Total.	Volatile.	Non-volatile.	Ratio of volatile to non-volatile.
		days	*cc.*	*cc.*	*cc.*	
Xylose.	41–11	3	123.7	64.6	59.1	100:91
	41–11	6	194.8	106.4	88.4	100:83
	41–11	8	207.2	115.2	92.0	100:80
	41–11	10	222.7	121.2	101.5	100:84
	41–11	14	226.5	124.0	102.5	100:83
	41–11	90	209.4	118.2	91.2	100:77
	118–8	3	126.8	66.6	60.2	100:90
	118–8	6	196.2	107.6	88.6	100:82
	118–8	8	224.7	130.4	94.3	100:72
	118–8	14	231.5	132.4	99.1	100:75
Arabinose.	41–11	28	234.8	136.6	98.2	100:72
	118–8	28	248.8	138.2	110.6	100:80
Rhamnose.	41–11	30	No fermentation.			
	118–8	30	“	“		

It will be noted from Table II that more than 12 per cent of normal acid was produced in a culture containing 2 per cent of xylose after 3 days incubation and nearly 20 per cent after 6 days. The maximum was found after 10 days of growth, about 22 per cent of normal acid. After 14 days incubation a portion of the culture was removed and the sugar content determined. In every case it was found that the bacteria had destroyed the pentose sugar.

The interesting fact noted from the data of this table is the relation between the volatile and non-volatile acids. This ratio is approximately 100 parts of volatile acid (cc. of 0.1 N acid) to 82 parts of non-volatile acid. It has been found that the acids formed consist almost exclusively of acetic and lactic acids. Arabinose apparently behaves like xylose, being decomposed rapidly into acetic and lactic acids. On the other hand, the methylpentose rhamnose is not attacked. Here it seems that the presence of the methyl group and the configuration of the hydroxyl groups prevented these organisms from decomposing it.

Fermentation of Glucose, Galactose, Mannose, and Fructose.

The aldo-hexoses, glucose, galactose, and mannose, are decomposed with the production of acetic acid, lactic acid, and ethyl alcohol, while from the ketose, fructose, mannitol is formed instead of ethyl alcohol. The total amount of acid formed, however, is not half that obtained from the pentose sugars, xylose and arabinose. The results of the fermentation of the hexose sugars are given in Table III.

Glucose.—Within 24 hours after inoculation the glucose cultures showed a well marked turbidity, a few gas bubbles, and a well defined acid reaction. The acidity with this hexose amounted to 10 to 14 per cent of normal acid, depending to a certain extent on the age of the culture. The general course of fermentation with glucose is very different from that noted with xylose and arabinose. Acids are formed rapidly in the early stages of fermentation, usually 2 to 3 days after inoculation; and then this period of rapid fermentation is followed by one of slow acid production. Apparently the pentose fermenters can make direct use of only a portion of the glucose. Of the 2 per cent present in the cultures at the beginning, it was found by analyses that more than half of this amount remained at the end of 30 days growth. The same incomplete consumption of sugar also occurs with galactose and mannose but, as already mentioned, to only a slight extent in the fermentation of xylose. Factors other than the accumulation of salts of acids must operate to produce this noteworthy difference in the behavior of the bacteria toward the hexoses. The sugar consumed correlates with the total acid formed which in the case of glucose is only half as great as that obtained from the fermentation of the pentoses, arabinose and xylose. Concerning the proportion between the volatile and non-volatile acids, the interesting fact is brought out that in the early stages of fermentation this ratio is about 100 to 500. Apparently this ratio changes in the older cultures, the volatile acid increases, while the non-volatile acid decreases. Although the

TABLE III.

Fermentation of Hexoses.

	Culture No.	Time.	0.1 N acid in 100 cc. of culture.			
			Total.	Volatile.	Non-volatile.	Ratio of volatile to non-volatile.
		days	*cc.*	*cc.*	*cc.*	
Glucose.	41–11	15	99.2	16.0	83.2	100:520
	41–11	30	106.5	26.3	80.2	100:305
	41–11	75	149.4	69.4	80.0	100:115
	118–8	15	98.0	15.8	82.2	100:520
	118–8	25	119.9	20.6	99.3	100:482
	118–8	30	100.7	18.6	82.1	100:441
	118–8	75	125.4	54.6	70.8	100:130
Galactose.	118–8	10	63.4	10.7	52.7	100:493
	41–11	30	77.2	16.7	60.5	100:362
	118–8	30	76.6	13.3	63.3	100:476
Mannose.	41–11	14	44.9	16.1	28.8	100:179
	41–11	35	90.7	54.7	36.0	100: 66
	118–8	14	33.9	16.9	17.0	100:101
Fructose.	41–11	14	88.5	52.0	36.5	100: 70
	41–11	21	91.2	53.4	37.8	100: 71
	41–11	28	97.0	60.0	37.0	100: 62
	41–11	42	133.5	72.5	61.0	100: 84
	118–8	14	89.5	51.9	37.6	100: 72
	118–8	42	133.9	74.0	59.9	100: 81
	118–8	4*	68.8	39.9	28.9	100: 72
	118–8	58*	69.7	49.7	20.0	100: 40

* Same culture analyzed after 4 and 58 days.

evidence is not conclusive, it seems that the non-volatile acid, lactic, is slowly converted into the volatile acid, acetic. The results of fermentation tests with the salts of lactic acid lend support to this assumption. In the pentose sugars no such change in the ratio of acids was noted.

Galactose.—The pentose bacteria ferment galactose in a manner almost identical with that of glucose. From the total acid produced it appears that galactose is somewhat less available than glucose.

Mannose.—This sugar is not fermented in the same way as glucose and galactose. As a matter of fact, mannose occupies a unique position among the hexose sugars whose fermentation has been studied. The decomposition of the mannose is effected much more slowly than is the case with glucose or galactose. The difficult fermentation of mannose may possibly be correlated with the difference in stereoisomeric structure from that possessed by the other aldo-hexoses. As may be seen from Table III, the total acid formed after 14 days is about 3 to 4 per cent of normal acid and the ratio about 100 parts of volatile to somewhat more than 100 parts of non-volatile acid.

Fructose.—Fructose is decomposed in a very different manner from any of the other hexoses. These organisms attack this ketone sugar vigorously, reducing it to mannitol and forming at the same time volatile and non-volatile acids and carbon dioxide. The soluble acid products of fermentation have been found to consist almost entirely of acetic and lactic acids. From sugar determinations it was found that the fructose is completely destroyed within 4 to 6 days after inoculation. It is clear from the results of Table III that the hexose sugars are fundamentally dissimilar in respect to availability; fructose is by far the most easily decomposed; glucose and galactose come next in order; and mannose last. It is significant that, with fructose, mannitol is formed as an intermediate product, and later this intermediate product is in turn decomposed with the formation of soluble acids. For more detailed information on this phase of the subject, see a later report.[7] The ratio of acetic acid to lactic acid in the case of fructose is about 100 to 70.

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[7] Peterson, W. H., and Fred., E. B., *J. Biol. Chem.*, 1920, xli, 431.

Fermentation of Sucrose, Maltose, Lactose, Raffinose, and Melezitose.

Next to the pentoses and hexoses in availability as measured by the production of acid come the disaccharides, sucrose, maltose, and lactose. Sterilization of the disaccharides may result in a partial hydrolysis and the production of the corresponding monosaccharides. A 2 per cent solution of sucrose in distilled water heated for 45 minutes at 15 pounds pressure showed a production of 0.012 gm. of reducing sugar per 100 cc. of solution, or 0.006 gm. of reducing sugar per gm. of sucrose. The same quantity of sucrose in yeast water gave 0.008 gm. of reducing sugar per gm. of sucrose. In order to obviate the influence of this reducing sugar, a sucrose solution was sterilized by passing through a Chamberland filter. The bacteria started somewhat more slowly in the latter case but the final acidity developed was essentially the same as in those cultures sterilized by heat. Of these three, sucrose appears the most available, giving an aver-

TABLE IV.

Fermentation of Disaccharides and Trisaccharides.

	Culture No.	Time.	0.1 N acid in 100 cc. of culture.			
			Total.	Volatile.	Non-volatile.	Ratio of volatile to non-volatile.
		days	*cc.*	*cc.*	*cc.*	
Sucrose.	41–11	25	117.8	53.4	64.4	100:121
	41–11	48	190.8	75.4	115.4	100:153
	118–8	25	86.8	29.4	57.4	100:195
	118–8	48	148.8	54.8	94.0	100:172
Maltose.	41–11	16	102.1	29.6	72.5	100:245
	118–8	16	105.6	29.5	76.1	100:258
Lactose.	41–11	25	82.2	33.7	48.5	100:144
	118–8	25	82.2	35.0	47.2	100:135
Raffinose.	41–11	20	No fermentation.			
	118–8	20	" "			
Melezitose.	41–11	20	" "			
	118–8	20	" "			

age of almost 17 per cent of normal acid after 48 days incubation. Maltose is fermented in much the same manner as sucrose, but was perhaps not so available. Too much emphasis should not be placed on the ratio between volatile and non-volatile acids, since the time of incubation was different for the two sugars, sucrose and maltose. Lactose is broken down very slowly by this group of bacteria, giving a much lower acidity than the other disaccharides. The trisaccharides, raffinose and melezitose, show little if any fermentation. Table IV gives the results of these fermentations.

TABLE V.

Fermentation of Alcohols and Glucoside.

	Culture No.	Time.	0.1 N acid in 100 cc. of culture.			
			Total.	Volatile.	Non-volatile.	Ratio of volatile to non-volatile.
		days	*cc.*	*cc.*	*cc.*	
Mannitol.	41–11	15	13.6	7.0	6.6	100:94
	41–11	40	81.6	43.8	37.8	100:86
	41–11	80	114.8	61.0	53.8	100:88
	41–11	140	148.9	75.7	73.2	100:97
	118–8	15	11.2	6.0	5.2	100:87
	118–8	40	79.2	40.2	39.0	100:97
	118–8	80	87.9	46.7	41.2	100:88
	118–8	140	140.0	73.0	67.0	100:92
Glycerol.	41–11	30	9.6	5.0	4.6	100:92
	118–8	30		5.0		
Salicin.	41–11	20	31.7	20.5	11.2	100:55
	118–8	20	25.9	17.1	8.8	100:51
Esculin.	41–11	20	No fermentation.			
	118–8	20	" "			

Fermentation of Mannitol, Glycerol, Salicin, and Esculin.

The polyatomic alcohol, mannitol, furnishes a fair source of carbon for the pentose-fermenting group of organisms. As shown in the results of Table V mannitol is fermented very slowly at first; after 140 days, however, the total acid reaches more than

14 per cent normal. The ratio between the two acids is maintained about the same throughout the entire fermentation period, usually 100 to 86–97. Glycerol was fermented very slowly. It is unsuitable as a source of carbon for these organisms. The glucoside, salicin, is slowly decomposed with the production of about two parts of volatile to one part of non-volatile acid. Esculin was not fermented.

Fermentation of Starch, Inulin, Cellulose, and Xylan.

The cultures containing the polysaccharides, starch, inulin, and cellulose, failed to show any decided increase in acids (Table VI). On the other hand, xylan decomposed very slowly, forming almost equal amounts of volatile and non-volatile acids.

TABLE VI.

Fermentation of Polysaccharides.

	Culture No.	Time.	0.1 N acid in 100 cc. of culture.			
			Total.	Volatile.	Non-volatile.	Ratio of volatile to non-volatile.
		days	*cc.*	*cc.*	*cc.*	
Starch.	41–11	30	No fermentation.			
	118–8	30	"	"		
Inulin.	41–11	30	"	"		
	118–8	30	"	"		
Cellulose.	41–11	30	Trace.	Trace.	Trace.	
	118–8	30	"	"	"	
Xylan.	41–11	60	58.7	27.5	31.2	100:113
	118–8	60		35.3	Lost.	

Fermentation of Salts of Organic Acids.

In order to determine the value of the salts of organic acids as a source of carbon, the fermentation of lactic, pyruvic, succinic, malic, tartaric, and citric acids was studied. In all cases the sodium or calcium salt of the acid was used. The salts of lactic, pyruvic, and malic acids were fermented, but no fermentation was found in the case of the succinic, tartaric, and citric acid salts. The sodium malate fermentation has been described in connection with the fermentation of fructose and results in the production of lactic acid and carbon dioxide as the chief products together with a small amount of acetic acid. In the case of the lactic acid salt, about 30 per cent of it was converted into acetic acid in 30 days. More complete quantitative data on the fermentation of the organic acids and the relation of these to the fermentation of the sugars will be considered in later papers.

SUMMARY.

The fermentation of various carbohydrates and related compounds was studied by means of the change in reaction and by gas production in fermentation tubes. It was found that the average limit of growth in the yeast water medium was approximately pH 3.6 to 4.0 in the case of xylose, glucose, galactose, fructose, sucrose, and lactose. Mannitol was decomposed more slowly and gave a limit of about pH 4.3. The results of acidity measurements fail to give any idea of the amount of the carbohydrate consumed. The fermentation tube method was tried and found valueless. Apparently the pentose fermenters break down the carbon compound with the production of gas, largely CO_2, which does not accumulate in the closed end of the fermentation tube. In the case of glucose and galactose as much as 27 per cent of this sugar was converted into CO_2 without any accumulation of gas.

In this work special emphasis was placed on the total acid formed from 2 per cent solutions of the various compounds as well as the amount of volatile and non-volatile acid. The pentose sugars, arabinose and xylose, decomposed rapidly forming more than 20 per cent of normal acid, divided into almost equal amounts of acetic and lactic acids. In less than 14 days the xylose and arabinose were completely destroyed. Rhamnose was not attacked by the pentose fermenters.

The aldo-hexoses, glucose and galactose, are decomposed in a similar manner, yielding acetic acid, lactic acid, and ethyl alcohol. However, the total amount of acid produced is small as compared with that from the pentose sugars; approximately 10 to 14 per cent of normal acid is formed. The non-volatile acid is produced in much larger amounts than the volatile; the ratio is about five parts of non-volatile acid to one part of volatile acid. Glucose is perhaps more easily fermented than galactose. The extent of fermentation is about the same with these two sugars; after 15 days approximately one-half of the sugar is consumed.

Mannose ferments more slowly than either glucose or galactose and yields nearly equal quantities of volatile and non-volatile acid.

The ketone sugar, fructose, is rapidly reduced by the pentose bacteria forming mannitol; and at the same time volatile and non-volatile acids and CO_2 are formed. The decomposition of the fructose is very rapid. Within 4 to 6 days after inoculation this sugar has completely disappeared.

The disaccharides, sucrose, maltose, and lactose, are all fermented, but never completely. Of these three substances, sucrose is the most available. Lactose, on the other hand, is broken down very slowly. The products of fermentation are chiefly volatile acid and non-volatile acid, with an excess of the latter. Raffinose and melezitose were not fermented.

The polyhydric alcohol, mannitol, is slowly broken down by the pentose fermenters forming almost equal amounts of the two acids, acetic and lactic. Glycerol and the glucoside, salicin, ferment very slowly, while another glucoside, esculin, fails to show any fermentation.

The polysaccharides, starch, inulin, and cellulose, are not fermented; xylan is decomposed very slowly.

The organic acids, succinic, tartaric, and citric, are not attacked; while pyruvic, lactic, and malic acids are fermented. From lactic acid, acetic acid is produced; and from malic, lactic acid, carbon dioxide, and acetic acid are the chief products.

A PHOTOMETRIC TURBIDIMETER.

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(Received for publication, March 3, 1920.)

Turbidimeters and nephelometers are instruments designed for practically the same purpose; that is, making quantitative determination on certain substances by estimating the translucency possessed by certain concentrations where a cloudiness exists or can be brought about. Some authorities call their instruments turbidimeters because they measure side scattered light, others call theirs nephelometers because they measure light reflected off at different angles (same phenomenon). The nephelometer is properly so called because it measures the amount of cloudiness in the substances, but the latest models of these instruments measure the amount of light that is transmitted through or is scattered by the cloud which exists in the substance.

In the case of either instrument, the operator arrives at his determination by measuring the amount of light that reaches his eye, whether or not he subtracts this from a total to get the absorbed light.

It occurred to the authors of this paper that in the use of the present instruments the operator is handicapped in two of the essential points. First, he must make a standard for comparison for almost every separate determination; and he must depend on the individual eye for the vanishing point of a light, or the merging of gratings. The present authors found that the latter differed with different eyes. In fact one of us found it differed with the right and left eye. Then with the use of the present instruments, where comparisons to standards are made, a point in the concentration is soon reached where the eye is entirely unable to distinguish between two substances that do not have a comparatively wide difference between them.

In 1900 Whipple and Jackson[1] designed the candle turbidimeter for the purpose of measuring turbidity of water. The candle flame under a certain depth of water would disappear. The turbidity of course was assumed to vary inversely as the depth required to cause the candle to disappear. This principle is used yet to a great extent in field work where electricity is unobtainable and other than arbitrary standards are inconvenient. This mechanism has been improved greatly by Sheppard[2] recently.

Our purpose has been to devise an instrument with which turbidities as great as that made by 1 cc. of a 5 per cent lemon oil extract in 100 cc. of water can be measured photometrically.

The latest models of nephelometers are not efficient to this extent. A history of the development of the nephelometer and a description of one of the later ones were published by Kober and Graves.[3] Dienert,[4] Bloor,[5] and Kober[6] developed the artificial light arrangement on the Duboscq colorimeter. In all these arrangements we must depend on the sensitivity of the eye for results.

Richards and Wells[7] provided aid for the eye in comparing intensities of illumination by means of ground glass wedges on their eyepiece. Kober and Graves[3] provided a sort of Lummer-Brodhun field for this comparison by the use of reflecting mirrors. A similar device was also employed by Marshall and Banks[8] in their instrument. But with all these instruments judgment depends on the ability of the eye to match intensities of light, and concentrations of substances soon go beyond that ability.

It seemed to the authors as though some photometric device such as the oil spot on paper would be more sensitive to slight differences in intensity of illumination.

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[1] Whipple, G. C., and Jackson, D. D., *Tech. Quart.*, 1900, xiii, 274.

[2] Sheppard, S. E., *J. Ind. and Eng. Chem.*, 1920, xii, 167.

[3] Kober, P. A., and Graves, S. S., *J. Ind. and Eng. Chem.*, 1915, vii, 843.

[4] Dienert, F., *Compt. rend. Acad.*, 1914, clviii, 1117.

[5] Bloor, W. R., *J. Biol. Chem.*, 1915, xxii, 145.

[6] Kober, P. A., *J. Biol. Chem.*, 1917, xxix, 155.

[7] Richards, T. W., and Wells, R. C., *Am. Chem. J.*, 1904, xxxi, 235.

[8] Marshall, J. T. W., and Banks, H. W., 3rd, *Proc. Am. Phil. Soc.*, 1915, liv, 176.

The apparatus which we have designed and operated, and which we wish to describe here, is fashioned so that a strong light is passed through a definite and comparatively narrow column of the substance in question, and what is not lost falls upon one side of an oil spot to balance with an amount that we permit to come from the opposite side by causing graduated losses on similar sources, under standard conditions. The apparatus consists of a dark box 12½ inches long, 4½ inches wide, and 8 inches high, having a dark compartment at each end for the standard lights, next to these the small dark compartments for the cell of unknown substances on the one side and the standards on the other, a very small compartment in the middle for the paper with its oil spot, and the narrow mirrors which reflect the images of the spot through a small eyepiece directly in front. Fig. 1 is a diagram of the apparatus.

The method of using this instrument will be readily seen. The unknown is put into the cell to the left as soon as prepared according to specified conditions and the eye is placed over the eyepiece in front. (Eyepiece is a wooden drawer knob with widening hole toward the screen and serves to lift lid.) Then switch on lights and turn the wheels, by the portion which extends above slots in lid, until the two intensities of illumination are balanced. Then read the resistance, units on first wheel to the right and tenths on the second. The visible numbers on the exposed portion of each wheel tell the equivalent of standard discs brought opposite the aperture through which the light passes. Each different substance determined has to have a scale of its own worked out previously. Then with the reading obtained, by comparing to the proper scale, percentages can be read.

Fig. 2 shows the construction of the wheels containing the standard discs.

While there might be no limit to the number of wheels with different denominations of discs, our instrument has only two. In our experience we found this to be sufficient for the low percentages that must of necessity be expressed. The percentages can, of course, be run to one or two decimals further either way by using varying amounts of the substance in solution.

In the compartment in which the unknown is placed, on the wall next to the light, a tube of the same size as the apertures for

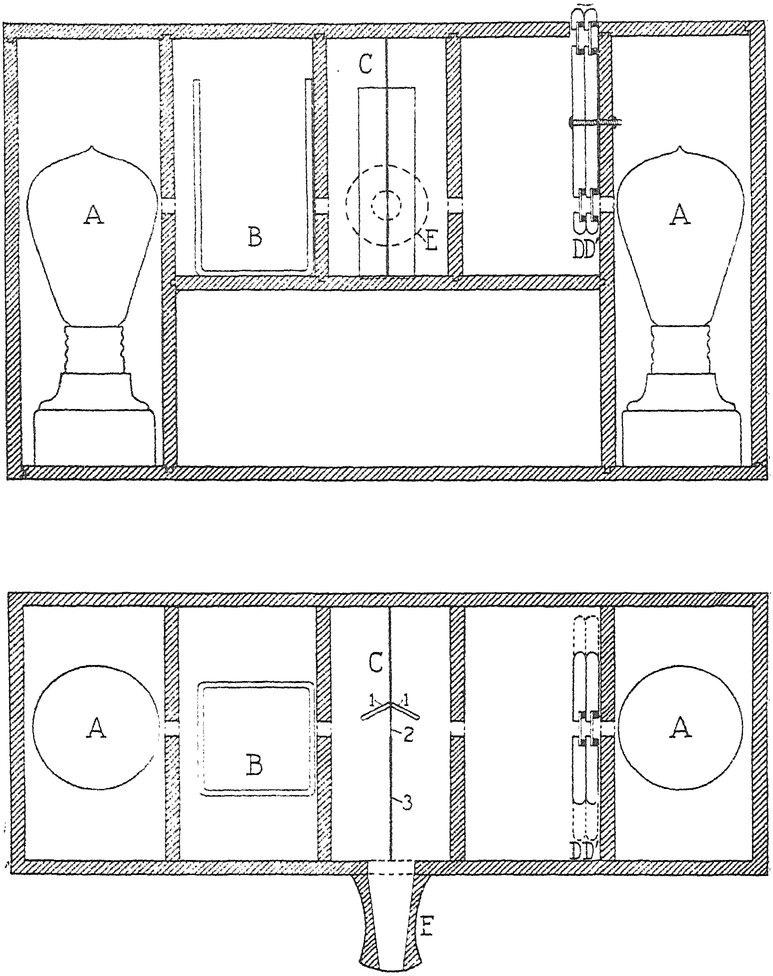

Fig. 1. Upper figure is a diagrammatic view from the side; lower figure view from above.

AA = 50 watt nitrogen lamps joined parallel.
B = Cell to hold substance to be tested.
C = Photometer, (1) reflecting mirrors, (2) oil spot, (3) screen of white paper.
DD′ = Discs holding standards of ground glass.
E = Eyepiece through which the photometer is viewed.

the light, and of a length equal to the thickness of the two wheels, is placed in the light aperture to give the same conditions as to confining the light rays. For special purposes the compartments for the unknown and the disc wheels should each be large enough to contain a standard cell 2 inches thick. However, an inch cell is what we used. When cells smaller than 2 inches are standardized and used they should always be placed in the same position against the wall next the screen compartment. When two cells are used they should be similarly situated in their respective compartments and the zero holes in the disc wheels brought into play.

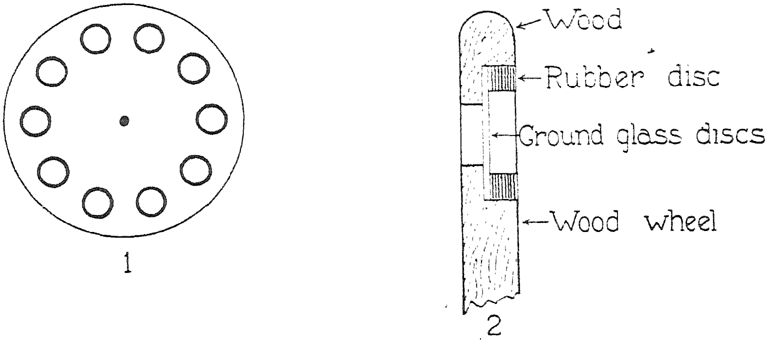

Fig. 2. (1) Lateral view of disc wheel; (2) cross section through discs.

To standardize the disc wheels for a certain substance, we have only to make one standard and place similar amounts in two cells, one for each side of the screen, and adjust the two lights to a zero reading by turning in discs or interchanging lights and turning in discs. Then certain standards which are multiples of each other are made and read and the readings are recorded on a slip which may be filed away to furnish a scale for that same substance in subsequent work. This saves making a new standard for each determination, provided the conditions of preparation are also standardized.

In the use of such an apparatus it is necessary to take cognizance of the work of Richards and Wells,[9,10] who in several articles call

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[9] Richards, T. W., and Wells, R. C., *J. Am. Chem. Soc.*, 1905, xxvii, 485. Richards, T. W., *Am. Chem. J.*, 1906, xxxv, 510.

[10] Wells, R. C., *Am. Chem. J.*, 1906, xxxv, 99.

attention to the existence of a progressive change in state of precipitates under different conditions. We can gather encouragement too from Kober[11] in his nephelometric determinations of ammonia, phosphorus, calcium, and the fats and oils; also from the work of Lamb, Carleton, and Meldrum[12] who standardized conditions for chlorine for the nephelometer.

In view of some facts recorded by Wells[10] we could not claim to be able to operate this instrument with all sorts of precipitates, but we hope that with several of the substances which have already been standardized for turbidimetry we can multiply the accuracy and save the time required for making new standards for each determination. We believe also that a great many more additions can be made to the list of substances determinable by the turbidimeter.

TABLE I.

SO_3 in 100cc. H_2O.	Readings by observers.			Average reading.
	A.	B.	C.	
mg.				
10	20	20		20
12.5	24	25	25	24.66
13	25	25		25
13.5	26	26		26
14	27	27		27
15	29	30	31	30
50	48		49	48.5

In order to test the efficiency of the instrument as compared to others we tried two different substances—chlorides as precipitated with $AgNO_3$ and $BaSO_4$ as obtained by 0.1 N H_2SO_4 and $BaCl_2$ in excess. We found that Whipple and Jackson,[1] Dienert,[4] and Muer[13] had operated the nephelometer and turbidimeter on sulfates in water. But Muer was able to determine within only 5 per cent of the true amount when the concentration reached 50 mg. of SO_3 to 100 cc. of water.

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[11] Kober, P. A., *J. Ind. and Eng. Chem.*, 1918, x, 556.

[12] Lamb, A. B., Carleton, P. W., and Meldrum, W. B., *J. Am. Chem. Soc.*, 1920, xlii, 251.

[13] Muer, H. F., *J. Ind. and Eng. Chem.*, 1911, iii, 553.

We worked out several different tables in the adjustment of our standards that proved the efficiency of the apparatus, but Table I represents results obtained by three different observers.

Observers A and B were the same persons each time. Observer C was a different person each time. If these were unknown substances supposed to contain sulfate and each case was assumed to be a 1 per cent solution of the unknown solid the average reading would be 1, 1.23, 1.5, and 2.43 per cent. The last, the one most in error, would be off 0.075 per cent. About 1 hour was occupied in the reading of each of these, each observer taking at least three sittings before giving his ultimate readings. At the end of each ½ hour for the following 1½ hours Observer A made observation on each preparation and found that they deteriorated at the rate of 0.1 per cent per ½ hour. We then made the 25 mg. standard with 25 cc. of glycerol and 75 cc. of water and made observations every ½ hour for 3 hours. We found that it then read 35 instead of the 24.66 reading obtained in the water suspension, that it remained 35 throughout six successive readings, and then fell at the rate of about 0.1 per cent each hour. We found in all readings that we could stir the precipitate just previous to taking the reading without changing the reading at all, and the above described deterioration occurred regardless of the stirring, and was doubtless due to agglomeration.

We operated the instrument then on AgCl preparations according to the method of Lamb, Carleton, and Meldrum[12] and found that it was only after working considerably with standards that we could compare light intensities with the yellow color on one side and white on the other. Then taking several readings made by three independent observers, each taking at least three sittings, we obtained the data given in Table II.

We note here that the concentration 2.65 mg. of chlorine to 100 cc. of solution, which was pronounced too concentrated by Lamb, Carleton, and Meldrum, was read the same by Observers A, B, and C and that one-fifth more than this concentration, which is about three times the concentration which they pronounced best, has not so great a deviation from the average as they had with the smaller concentration.

After taking the above reading we performed a few experiments placing fractional portions of the colored substance on the standard side of the instrument and making up the balance by turning of discs. We got very good results with a much higher concentration than was used in Tables I and II.

We believe that with an instrument in which the difference between a standard and an unknown can be made up with known additions to the weaker one, as is the case with the Marshall-Banks instrument and the one we are here describing, that the above scheme will not only save the making of standard substances to compare to the unknown but it will serve the purpose better. It seems to avoid errors that might creep in on account of the unknown's having more of some interfering substance than does the standard. We are now working out more definite data in this connection and also working on the problem of neutralizing the color or other interfering conditions by bringing colored substances or colored glasses into the path of the light, and reducing the light on the side of the standard[14] with a rheostat resistance in order to obtain more accurate reading in the presence of colors. We hope also that ultimately a colorimeter may be made of this same apparatus.

TABLE II.

Chlorine in 100cc. water and alcohol.	Readings by observers.			Average reading.
	A.	B.	C.	
mg.				
2.65	25	25	25	25
3.18	30	30	31	30.33
6.36	58			58
10.0	90			90

SUMMARY.

The photometric oil spot is applied to the turbidimeter to balance intensities of illumination, in measuring turbidities. A device is made in which thin columns of substance can be used and a system of standards is arranged.

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[14] Suggested by Professor W. F. Sudro of this laboratory.

THE CHEMICAL ISOLATION OF VITAMINES.

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(Received for publication, February 3, 1920.)

The existence of at least three vitamines, namely the antiscorbutic, the so called fat-soluble, and the antineuritic, is known at the present time. This paper deals principally with the antineuritic vitamine.

The important stage in the chemical investigation of this substance began with the classical research of Eijkman in 1897, who was able to show that rice polishings contain the antineuritic vitamine and that this substance is dialyzable and not precipitated from its solution by alcohol. Eijkman's work stimulated Funk to attempt the chemical isolation of the substance. In a series of researches he established the following facts. First of all he found that the curative substance is of a simple nature, as yeast can be hydrolyzed for 24 hours with 25 per cent sulfuric acid without causing the destruction of its vitamine. The active substance is precipitated by phosphotungstic acid and is mainly found in the silver nitrate-baryta fraction, when subjected to silver precipitation. This fraction contains three classes of biological products; namely, the histidine, pyrimidine, and nicotinic acid groups. On working up these fractions from yeast Funk isolated three substances which on analysis yielded the following formulas: $C_{24}H_{19}O_9N_5$, $C_{23}H_{23}O_9N_5$, and $C_6H_5O_2N$ (nicotinic acid). By using the same or similar methods, Suzuki, Shimamura, and Odake, Vedder and Williams, and Edie, Evans, Moore, Simpson, and Webster were also able to isolate more or less active vitamine fractions from active raw material. Similar fractions were obtained by Funk from milk, brain, lime juice, rice polishings, and cod liver oil.

The fact that the antineuritic vitamine is precipitated from its solution by reagents, which as a rule precipitate basic substances, and the observation that the active, purified fraction contained nitrogen led Funk to the adoption of the term vitamine, a terminology which future work may perhaps prove to be incorrect from the standpoint of chemical nomenclature.

The work of the chemical isolation of vitamine up to 1913 had thus lead to a considerable increase in our knowledge of the chemical character of the antineuritic vitamine, but satisfactory methods for the preparation of the substance in a pure form and in large amounts were still lacking. In 1913 the United States Public Health Service extended its investigations of the etiology of pellagra on account of the menacing increase in this disease in the southern states. One of us (Voegtlin) began a study of the nutritional conditions in these districts, followed early in 1914 by a chemical study of the possible etiological factors of this disease. That the disease might be due to the deficiency of certain factors of the vitamine type as suggested by Funk had been early recognized by Voegtlin, who undertook extensive animal experiments in this connection. With the same purpose in view, work was also begun in this laboratory on new methods for the preparation of highly active vitamine preparation in relatively large quantities for the treatment of pellagra. As the first result of this work, Seidell succeeded in obtaining a very active preparation by treating filtered autolyzed yeast with fullers' earth. This active preparation can be obtained easily on a large scale and was used for the treatment of pellagra patients by Voegtlin, Neill, and Hunter.

In the experimental work, part of which the authors of the present paper are about to describe and which covered a period of several years, brewer's yeast was also made use of as a raw material for the antineuritic vitamine. Nearby breweries furnished an ample supply of bottom yeast, which was pressed into a cake by means of a filter press before additional fermentation had taken place.

The yeast was then placed in a hot room (40°C.), in alcohol barrels as containers, and allowed to autolyze. 200 cc. of chloroform were used for every 100 pounds of yeast. Autolysis was usually complete in about 36 hours, at which time the thin liquid was run through the filter press yielding a clear dark liquid with a specific gravity of about 1.01. This liquid was used as the source of material in the early work, even though it was finally found to be very unsatisfactory on account of its chemical complexity.

Extraction with Olive Oil.

When autolyzed yeast filtrate is treated with concentrated hydrochloric acid, a heavy, flocculent precipitate is obtained. It was found in connection with the yeast filtrates used in this laboratory that 40 cc. of concentrated acid were necessary for complete precipitation of 1 liter of filtrate. This material is easily filtered off and amounts to about 4.35 per cent of glue-like material, giving the common protein reactions. In all the work which is described in the following pages, the yeast filtrate was treated in this manner. It was found that this filtrate could be kept for a period of at least 2 years without losing its physiological activity, that it can be used for oral administration in the treatment of polyneuritis by neutralizing the acid with sodium hydroxide, and that it represents a suitable raw material for adsorption isolation experiments when the hydrogen ion concentration is adjusted by means of sodium borate.

In view of the fact that in nature the antineuritic vitamine is closely associated with lipoids, the idea suggested itself that this vitamine might be soluble in fats.

Autolyzed yeast filtrate was shaken with olive oil on a shaking machine until an emulsion was formed, with 1 cc. of olive oil for each 4 cc. of yeast filtrate. The emulsion was allowed to stand until two distinct layers were formed and by means of a separatory funnel the oil layer was separated. The oil was filtered to remove a small amount of sediment and then taken up in eight or ten volumes of ether. 0.1 per cent hydrochloric acid was used for the extraction of the ether solution. The acid extract was slightly pigmented and was concentrated in vacuum. A heavy precipitate was obtained with phosphotungstic acid, giving a deep blue color after the addition of sodium carbonate. An insoluble precipitate is formed with picric acid. The biuret test is negative. The extract promptly relieves the polyneuritic symptoms of pigeons.

The same results are obtained by the use of oleic acid instead of olive oil.

Dried Yeast.

After several unsuccessful attempts to remove impurities from the products obtained by extraction, the following method was used with a considerable degree of success. Dried brewer's yeast was selected as the source of material. The yeast as it was obtained from the brewery was pressed until it crumbled easily between the fingers. It was broken into small particles and dried with a current of air at ordinary temperature, which required 36 to 48 hours. This dried product retains its activity for many months if stored in a dry place.

Dried yeast prepared in this way was ground in a mill repeatedly until a very fine powder was obtained. This was placed in a balloon flask of suitable size with reflux condenser, and extracted with 95 per cent methyl alcohol in the proportion of 2 cc. of alcohol for each gm. of yeast. 1 cc. of concentrated hydrochloric acid was added for each liter of alcohol used in the extraction. The contents of the flask were heated to boiling on the water bath for 3 hours and the soluble part was filtered off by means of suction. The residue was washed once with 1 cc. of hot methyl alcohol per gm. of yeast. The yeast was then extracted a second time using the same proportions of solvent. The extracts were combined and the alcohol was removed in vacuum at 35°C. The wax-like residue was repeatedly extracted with ether and 0.1 per cent hydrochloric acid, the volume being kept as small as possible. The final acid extract should never exceed 2 cc. for each gm. of yeast. The acid aqueous extract is always tested on polyneuritic birds for its activity and then it is purified as described below.

Hot aqueous silver acetate is added to the extract until precipitation is complete by testing a small portion in a test-tube. This purine precipitate, which also contains $AgCl$, is carefully washed with distilled water. Only a small amount of the active material passes over into this purine fraction.

A large excess of silver acetate was then added to the filtrate from the purine precipitate, followed by saturated barium hydroxide solution until the mixture was distinctly alkaline to litmus. The precipitate was filtered and carefully washed with cold distilled water. Considerable precipitate is produced in this manner, and additional precipitate may be obtained by further addition of baryta.

The silver-baryta precipitate containing the vitamine and some other extraneous material was suspended in water and made distinctly acid with sulfuric acid. The precipitate was then decomposed with hydrogen sulfide with occasional stirring in order to break up any lumps. A slightly pigmented filtrate was obtained after removing the silver sulfide. The excess hydrogen sulfide was removed in vacuum and the filtrate was then treated with a slight excess of lead acetate to remove the sulfuric acid. The lead was removed by hydrogen sulfide and the filtrate concentrated in vacuum at 35°C., with a small amount of ethyl alcohol to aid the distillation. Up to this stage practically none of the activity is lost. This concentrated solution of vitamine and impurities was then treated with mercuric sulfate prepared according to the directions of Kossel and Patten. A pale yellow precipitate was formed upon the addition of the mercuric sulfate, which was filtered off and washed with a small amount of ice water. This precipitate represents the histidine fraction and does not contain active material. The filtrate containing an excess of mercuric sulfate was treated with absolute ethyl alcohol until precipitation was complete. This precipitate was pale yellow and contained the bulk of the active material. The degree of separation in this last procedure is influenced by two factors, (1) the concentration of excess mercuric sulfate present and (2) the final concentration of ethyl alcohol. If these adjustments are right, none of the active material remains in the alcoholic filtrate. The alcohol-insoluble precipitate was suspended in water and the mercury was removed by means of hydrogen sulfide. The filtrate from the mercuric sulfide was freed of hydrogen sulfide in vacuum. The sulfuric acid was removed by means of lead acetate, and the excess lead by hydrogen sulfide.

By eliminating the first lead treatment the process might be shortened, but it was found that this would lead to considerable pigment passing over into this fraction.

This solution was concentrated in vacuum at a low temperature. This fraction gives a purple solution when tested with ninhydrin. Phosphotungstic acid gives a heavy precipitate, slightly soluble in an excess of the reagent, with only a slight blue color upon the addition of saturated sodium carbonate. With diazotized sulfanilic acid and sodium carbonate a reddish brown color was obtained. The biuret test was negative. The solution does not yield a precipitate with picric acid. The solution was highly active when tested on polyneuritic birds. If the solution is concentrated in vacuum, over soda lime, a definitely crystalline product is obtained which shows activity as long as the crystals are surrounded with the mother liquor. The material was soluble in methyl alcohol and yielded a soluble hydrochloride. As soon as the crystals are washed with absolute ethyl alcohol and dried, the physiological activity is lost and the crystal form changes from spindles to prisms. When the prisms are redissolved in a relatively large volume of water and again allowed to crystallize, the spindle-shaped crystals are reformed. It seems probable that there are at least two substances in the final solution both of a distinctly basic character. Work is being continued on this subject, the material being tested as a protective product. It is realized that the activity may be in the non-crystallizable mother liquor as well as in the crystals.

One of the impurities is a histamine-like substance, as shown by the positive Pauly reaction. Voegtlin and Myers have recently shown that the active fractions possess physiological activity when introduced intravenously into dogs. There is a fall of blood pressure and a stimulation of pancreatic and bile secretion following the injection of this material. It is possible that this stimulating action on pancreatic secretion and the fall in blood pressure are actually due to the presence of traces of histamine or a histamine-like substance in these fractions, as according to Abel and Kubota histamine is very widely distributed in animal tissues and probably also in vegetable cells.

SUMMARY.

Autolyzed yeast filtrate on account of its complexity represents an unsatisfactory material for the chemical isolation of the antineuritic vitamine. Mastic, Lloyd's reagent, and ferric phos-

phate, in experiments which, because of their negative outcome, it has not seemed desirable to detail, have been found unsatisfactory adsorbing reagents because they lack specificity. These reagents remove also inactive basic material which cannot be separated by our present methods from the active material. Olive oil and oleic acid remove the antineuritic substance from autolyzed yeast filtrate, thus showing that it is fat-soluble as well as water-soluble in the form of a crude extract. Stachhydrine, trigonelline, and allied betaines show no antineuritic activity. Histidine and its esters are likewise inactive.

The active material is readily extracted from dried yeast by means of acid methyl alcohol. It can be purified by use of the Funk silver method and the mercuric sulfate procedure, yielding an apparently crystalline active substance. This substance becomes inactive upon drying and it is believed that impurities still remain which can be removed with additional modifications of the method described in this paper. The present method eliminates purines, histidine, proteins, and albumoses, leaving a liquid that can be crystallized and probably contains histamine or histamine-like substances. The physiological action of the active fractions resembles that of extracts obtained from the mucosa of the small intestine, when the intestinal and yeast extracts are purified in the same manner.

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OSMOTIC PRESSURE OF SERUM AND ERYTHROCYTES IN VARIOUS VERTEBRATE TYPES AS DETERMINED BY THE CRYOSCOPIC METHOD.

WITH A NOTE ON THE EFFECT OF FREEZING ON THE ARTERIAL BLOOD OF THE RAT.

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It is generally assumed that the osmotic pressure of animal cells is approximately equal to that of the body fluids which bathe them. Thus according to Mathews (1), "Animal cells presumably have an osmotic pressure approximately equal to that of the circulating liquids like the blood."

It would appear that the blood itself is an ideal tissue in which to study relations in osmotic tension between the cells and the surrounding fluid medium, the plasma, or in the case of defibrinated blood the serum. The results of a series of determinations of the freezing points of whole blood (defibrinated), blood serum, and corpuscles of various vertebrate types are recorded in Table I. In this series of papers we have accepted the assumption that the difference, Δ, between the freezing points of a solution and that of water is a measure of the osmotic pressure of the solution.

The blood samples were obtained in various ways, but in every instance the sample corresponded to the arterial type, before the readings were taken. In the case of the sheep the samples were drawn from the external jugular vein of the normal animal. The samples of dog blood were taken from the femoral vein while the animal was under light ether anesthesia. Ox blood and pig blood were obtained from the abattoir at the time the animals were bleeding freely from the carotid artery and jugular vein. Samples were obtained from rabbits, birds, and turtles by bleeding from the carotid, the animal being first stunned by a blow on the head. The samples of human blood were taken from the median basilic vein. All samples were either aspirated directly into a thick walled, clean, dry, pressure filter, receiving flask containing clean, dry, glass beads, or else caught in the same by allowing the blood to trickle through a paraffined glass funnel. The samples were

TABLE I.

Type.	Δ serum.	Δ defibrinated blood.	Δ corpuscles.	Difference between Δ serum and Δ corpuscles.
	°C.	°C.	°C.	°C.
Sheep	0.621	0.599	0.582	0.039
	0.575		0.538	0.037
Rabbit	0.615	0.585	0.570	0.045
	0.665	0.625	0.607	0.058
Dog	0.647		0.581	0.066
	0.630	0.585	0.605	0.025
Cat	0.68		0.605	0.075
	0.69		0.645	0.045
Man	0.585	0.555	0.520	0.065
	0.655		0.617	0.038
	0.615	0.586	0.563	0.052
Pig	0.600	0.585	0.575	0.025
	0.605	0.585	0.575	0.030
	0.601	0.585	0.582	0.019
Rat	0.619		0.582	0.037
Kitten	0.636		0.612	0.024
Ox	0.598	0.568	0.547	0.051
Fowl	0.650	0.640	0.630	0.020
	0.652		0.618	0.034
Pigeon	0.633		0.590	0.043
	0.690		0.621	0.069
Turtle	0.522		0.480	0.042

shaken in the open flask for about 5 minutes. In those instances in which the Δ of whole blood was determined, the sample for this purpose was taken at this point. The defibrinated blood was at once centrifuged, the serum and corpuscles were pipetted off into clean, dry tubes, and readings taken immediately. When blood samples were obtained at the abattoir a delay of about 2 hours occurred between the time of sampling and defibrinating, and the centrifuging. In as far as the Δ of a physiological fluid is a reliable index of the osmotic pressure of the same, it follows from the results of this series of experiments that the osmotic pressure of the contents of the red cells is in all instances lower than that of the corresponding serum, while that of the whole blood falls between these two values. Stewart (2) found that the Δ for the whole blood was 0.628°, that for corpuscles 0.597°C. Moore and Roaf (3), working with the blood of the pig, found that the Δ for the serum was always slightly higher than that of the erythrocytes. Their results are given in Table II.

TABLE II.

Experiment No.	Δ serum.	Δ corpuscles.
	°C.	°C.
1	0.607	0.577
2	0.539	0.519
3	0.591	0.573
4	0.586	0.570

They also found that when pig corpuscles were allowed to come into equilibrium with various salines, and afterwards separated from the same by centrifuging, the Δ for the cells was always in excess of that of the saline. The result of one such experiment by these authors follows.

	°C.
Δ original saline solution	0.983
Δ after admixture and centrifuging	0.847
Δ corpuscles after admixture and centrifuging	0.788
Difference	0.059

The results which are reported in Table I would therefore corroborate the findings of Moore and Roaf (3), as regards the variation in osmotic tension between the red cells and serum of the pig. This difference in osmotic pressure between the erythrocytes and the serum, which in the case of the pig blood was found to be comparatively small, varies considerably in the different types studied.

The maximum difference found between the Δ for serum and corpuscles is 0.075°C. in the case of cat, the minimum being 0.019°C. in pig. The average difference in the twenty-two experiments quoted is 0.043°C. Assuming that differences in Δ represent actual differences in osmotic pressure, the above average difference in Δ would present a difference of approximately 400 mm. of mercury between the osmotic pressure without, and within, the red cell. The depression of the freezing point of the centrifuged corpuscles was found in no instance to be equal to or greater than that of the corresponding serum. This being the case it is therefore established, providing always that the cryoscopic method is a reliable index of osmotic pressure, that there is a definite and appreciable difference in the osmotic pressure of the red cells and the serum of the types studied. One would seem justified also in concluding from the data furnished by these experiments that this variation in osmotic pressure between red cells and serum, which are apparently in osmotic equilibrium, is general throughout the vertebrate class.

It was found that laking of the corpuscles by repeated freezing and thawing did not have any appreciable effect on the Δ unless, however, one started with venous blood, in which case the depression was less due to loss of carbon dioxide but after equilibrium was established the reading remained constant. A typical example of this is quoted.

	°C.
Sheep blood, red cells (venous) first freezing	0.562
“ “ “ “ “ laked by alternate freezing and thawing, and agitated for 15 minutes with stirrer.	0.536

The effect of the carbon dioxide content on the freezing point was also tested in another manner. Blood drawn from the jugular vein of a sheep was divided into two portions after defibrination. Oxygen gas was bubbled through the one sample for 15 minutes, and through the other carbon dioxide gas was passed for a like period of time. The serum and corpuscles were then separated by centrifuging and the depression of the freezing point of the respective serum and corpuscles was determined. The results are as follows:

		°C.
Sample made arterial by oxygen gas	Cells	0.59
	Serum	0.64
" " venous by carbon dioxide gas	Cells	0.65
	Serum	0.70

Kovács (4) has previously shown that the addition of carbon dioxide to rabbit blood raised the Δ from 0.60 to 0.72°C.

The effect of freezing at all temperatures from −6 to −40°C. of oxygenated corpuscles was found to be negligible.

It is possible that the differences in osmotic pressure between serum and corpuscles which are indicated by these cryoscopic studies do not exist under the conditions holding in the blood stream of the normal animal.

The ease with which crystalline oxyhemoglobin of the rat blood can be obtained was constantly demonstrated in the freezing of the erythrocytes of this animal. While there was slight tendency for the oxyhemoglobin of other bloods to crystallize on freezing, it was found that in the case of the rat cells practically the whole mass of oxyhemoglobin crystallized out with a single freezing, and that, instead of showing the characteristic laky appearance on thawing, the rat cells were pinky red in color as viewed in the glass container. Microscopic examination revealed the fact that almost complete crystallization of the oxyhemoglobin had occurred, many crystals being contained in the red cell envelopes. Rat erythrocytes which had been frozen could be mixed with several volumes of distilled water with only a trace of hemolysis being manifested, and the bulk of oxyhemoglobin crystals could be recovered again by centrifuging or allowing sedimentation to take place. Rat cells made venous in type by bubbling carbon dioxide gas through them previous to freezing were laked in the usual manner.

SUMMARY.

The results of the determination of the depression of the freezing points of whole blood, blood serum, and corpuscles in various vertebrate types are recorded. The Δ for corpuscles is always found to be lower than for the corresponding whole blood or serum.

The average difference in osmotic tension between the red cells and the serum in twenty-two determinations as calculated from the data obtained by the cryoscopic method is approximately 400 mm.. of mercury.

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EFFECT OF DILUTION ON THE OSMOTIC PRESSURE AND THE ELECTRICAL CONDUCTIVITY OF WHOLE BLOOD, BLOOD SERUM, AND CORPUSCLES.

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It has been shown by the author in Paper I that the osmotic pressure of the red cells is appreciably lower than that of the serum in all bloods studied. In the present paper there are reported a number of experiments in which the effect of dilution on the osmotic tension, and on the electrical conductivity of whole blood (defibrinated), blood serum, and corpuscles has been studied.

The effect on the Δ of blood serum, whole blood, and blood corpuscles of dilution with an equal volume of distilled water is shown in Table I. It is found that, whereas in the case of blood serum the value for the Δ is decreased approximately 50 per cent as a result of twofold dilution, in the case of the corpuscles twofold dilution causes a decrease of from 57 to 60 per cent in the Δ. The effect of twofold dilution of whole blood was determined in a few cases only. The decrease in the depression of the freezing point of whole blood diluted with an equal volume of distilled water is greater than in that of the serum similarly treated, but less than that of the corpuscles diluted twofold.

It might be expected that in the case of a weak solution of electrolytes twofold dilution would result in the reduction of the osmotic tension of a unit volume to slightly over one-half the tension of the original solution. The increase in the degree of the dissociation as a result of dilution would cause a relative increase in the number of ions and thus the tension of the diluted solution would be more than half that of the original solution. The results indicate that blood serum behaves somewhat in this manner and probably whole blood and corpuscles do also, although the primary survey of the readings would not so indicate. As blood corpuscles contain approximately 30 to 40 per cent total solids, it is evident that when one dilutes 100 volumes of red cells with 100 volumes of distilled water, one obtains not a twofold dilution of the fluid fraction of the corpuscles but a much greater one. Thus if we assume that 25 per cent of the corpuscle is, as it were, framework and 75 per cent liquid, then on diluting 100 cc. of red cells with 100 cc. of distilled water one actually dilutes the 75 cc. of liquid in the cells to 175 cc., so that the dilu-

TABLE I.

Type.	Δserum.	Δserum diluted twofold.	Δdefibrinated blood.	Δdefibrinated blood diluted twofold.	Δcorpuscles.	Δcorpuscles diluted twofold.	x
	°C.	°C.	°C.	°C.	°C.	°C.	
Man	0.615	0.337	0.586	0.265	0.563	0.244	76.5
Sheep	0.608	0.309			0.520	0.223	76.0
Rabbit	0.615	0.320	0.585	0.277	0.570	0.240	72.7
	0.625	0.318			0.588	0.254	77.8
Dog	0.647	0.327			0.581	0.254	77.6
	0.630	0.330			0.585	0.255	77.2
Cat	0.685	0.345			0.605	0.255	72.8
Pig	0.600	0.315			0.575	0.242	72.6
	0.601	0.315	0.585	0.265	0.582	0.245	72.1
Rat	0.619	0.310			0.582	0.232	69.1
Kitten	0.636	0.322			0.612	0.259	73.3
Ox	0.598	0.306	0.568	0.276	0.547	0.220	67.2
Fowl	0.652	0.330	0.618	0.295	0.618	0.242	64.3
3 pigeons	0.690	0.343			0.621	0.262	73.0
	0.633	0.343			0.590	0.248	72.5
Turtle	0.522	0.255	0.512	0.231	0.480	0.192	66.6

tion is 3 to 7. If one does not allow for increase in the degree of dissociation of the electrolytes with twofold dilution or shrinking in actual volume occupied by the non-liquid portion of the red cell then the following formula should apply $\Delta_1 = \left(\frac{100 + x}{x}\right)\Delta_2$

where x = actual liquid in the red cell,
Δ_1 = depression of freezing point for red cells, and
Δ_2 = depression of freezing point for red cells diluted with an equal volume of distilled water.

When this arbitrary formula is applied to the results recorded for Δ's of red cells before and after dilution for purposes of comparison, one finds that the value for x varies from 66.6 per cent in the case of turtle cells to 77.8 per cent in the case of rabbit cells, while in the case of most of the non-nucleated types of red cells the value for x approximates closely to 75 per cent. Irrespective of in what state one may consider the protoplasm of the red cell to be (fluid, semirigid, or rigid), there is a certain fraction of its bulk which may be considered as framework, and it is the total bulk minus this factor which is in osmotic equilibrium with the surrounding serum. This latter fraction is approximately 75 per cent of the volume of the red cell. The framework (the term used advisedly) would include the cell membranes, the stroma, and, what would be most important, the disperse phase of the colloidal complex within the cell. The liquid phase or the dispersion medium might be considered as holding in solution the molecular and ionic dispersoids contained within the cell membrane with the exception of any such portion of the latter class which may be present in the adsorbed state.

The electrical conductivity of whole blood, blood serum, and erythrocytes of various vertebrate types both undiluted and diluted to various degrees was determined. The Kohlrausch method was employed. Readings were taken at 37°C. and the specific conductivities calculated. The figures given in Table II are for specific conductivities multiplied by 10^5.

As has been shown by Moore and Roaf (1) and others, the specific conductivity for serum is several times that for corpuscles. This is due very largely to the mechanical effect of the want of homogeneity of the fluid in the electric field, as shown by Moore and Roaf (1). The fact that the specific conductivity for cor-

TABLE II.

Specific Conductivities at 37° C.

Type.	Undiluted.	Degree of dilution.				
		2-fold.	4-fold.	8-fold.	16-fold.	32-fold.
Human serum	1,582	857	450	242	129	65
“ defibrinated blood	693	441	294	184	103	62
“ corpuscles	47	145	154	119	71	43
Cat serum	1,714	964	505	270	140	71
“ defibrinated blood	873	475	330	194	109	59
“ corpuscles	94	137	187	133	71	43
Dog serum	1,543	869	475	242	126	64
“ defibrinated blood	475	338	254	160	92	52
“ corpuscles	45	124	167	124	76	47
Rabbit serum	1,542	845	457	242	125	65
“ defibrinated blood	616	470	300	220	114	62
“ corpuscles	82	160	208	152	96	54
Sheep serum	1,582	869	471	242	126	64
“ defibrinated blood	893	546	346	197	106	58
“ corpuscles	133	206	206	131	87	47
Pig serum	1,582	857	463	240	125	63
“ defibrinated blood	725	514	331	187	110	59
“ corpuscles	75	192	229	158	80	48
Fowl serum	1,648	884	494	257	131	67
“ defibrinated blood	694	605	350	212	110	60
“ corpuscles	69	140	111	111	65	43
Pigeon serum	1,581	833	460	252	131	66
“ defibrinated blood	585		363	199	112	62
“ corpuscles	60	147	173	147	90	46
Turtle serum	1,323	685	369	198	102	53
“ defibrinated blood	822	514	301	176	93	48
“ corpuscles	81	171	182	143	78	44

puscles diluted several-fold with distilled water is always lower than the specific conductivity for serum or defibrinated whole blood at the same dilution would suggest that the ionic concentration within the corpuscle is less than that in the serum. It may be possible, however, as Moore and Roaf (1) have pointed out, that due to the adsorption of crystalloid by the colloid of the hemolyzed red cells the movement of the ions in the electric field is so inhibited that the conductivity is much lower than it would be if the same electrolytes were held in solution in an equal volume of distilled water. What is probably of greater significance, however, is the viscosity factor. This unfortunately could not be determined. The viscosity of the diluted corpuscles would

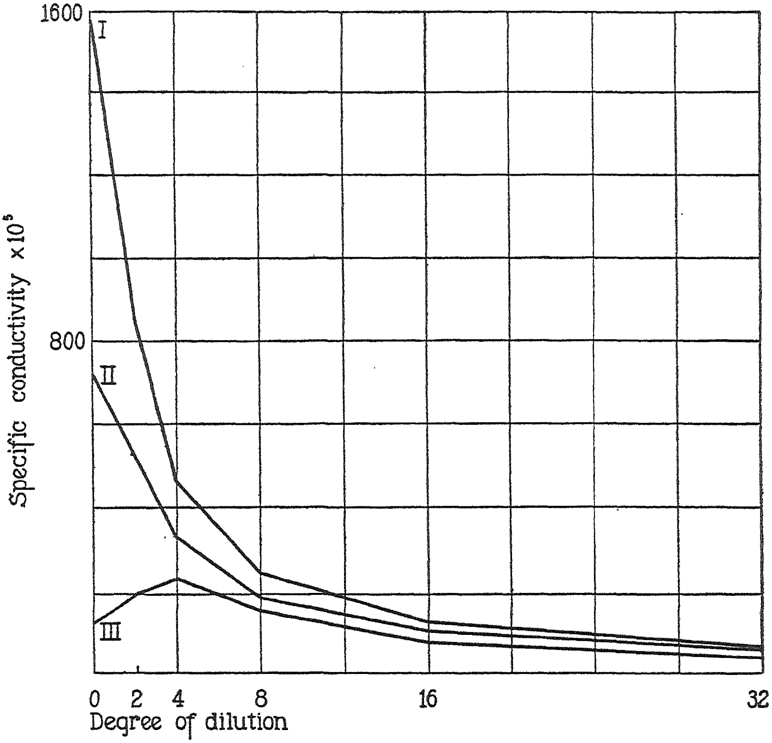

FIG. 1. Pig blood at 37°C. Curve I = serum; II = defibrinated blood; III = red cells.

no doubt be in the earlier stages of dilution appreciably higher than that of the sera of equivalent dilution.

The percentage of inorganic matter, exclusive of iron, in the red cell was found by Schmidt (2) to be lower than that in the serum, 1,000 gm. of corpuscles yielding 7.128 gm. of ash, while 1,000 gm. of serum gave 8.574 gm. of ash. Taking into account the specific gravity, 1.088 in the case of red cells, 1.029 in the case of serum, these figures would be corrected on the basis of percentage volume to 7.74 gm. of ash per 1,000 cc. of corpuscles and 8.82 gm. of ash per 1,000 cc. of serum—still a marked difference.

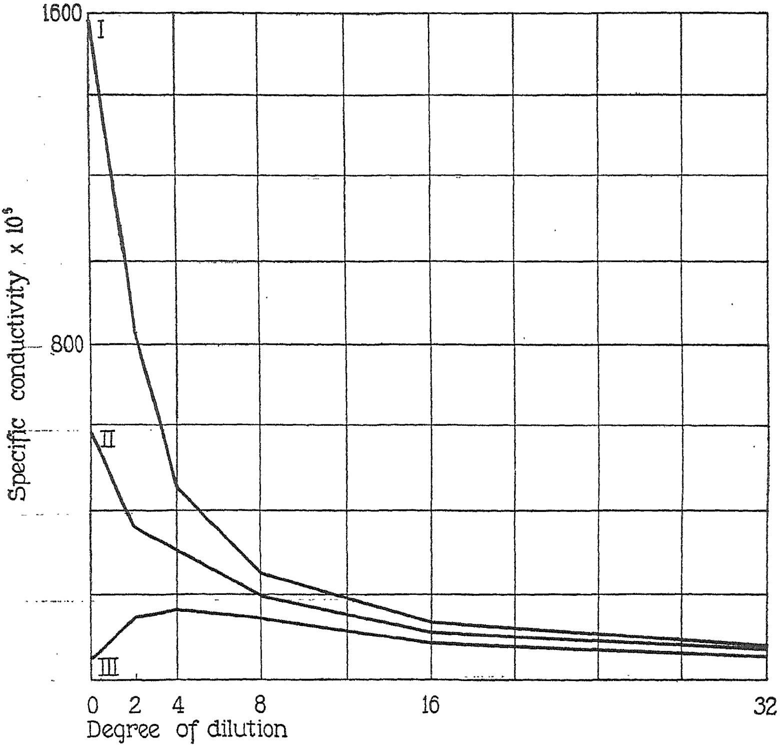

FIG. 2. Pigeon blood at 37°C. Curve I = serum; II = defibrinated blood; III = red cells.

Results of quantitative analyses such as these would favor the view that the total electrolyte content of the erythrocytes per unit volume is less than that of the serum.

If we take into account the point urged earlier in this paper that a certain definite portion of the red cell protoplasm may be considered as framework and, as such, actually reduces the fluid volume to approximately 75 per cent of the total bulk of the red cell, then it would follow that the concentration of electrolyte in this fluid must be equal to, if not greater than, that in the serum, unless an appreciable part is in a state of adsorption at the membrane surface or with colloidal aggregates.

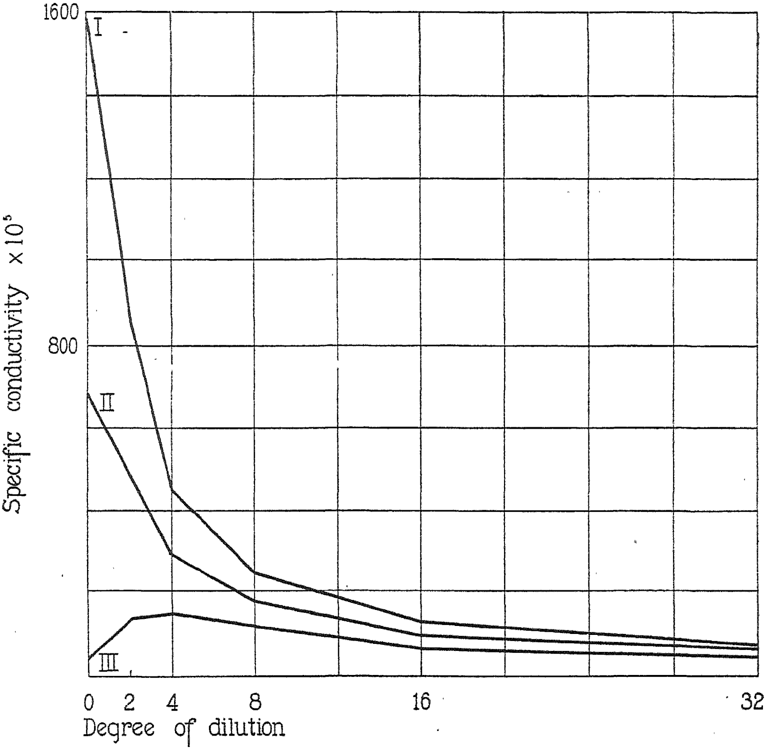

FIG. 3. Human blood at 37°C. Curve I = serum; II = defibrinated blood; III = red cells.

Preliminary freezing, and thus laking, of the corpuscles causes an increase in their conductivity, as Moore and Roaf (1) have shown. Preliminary freezing prior to twofold dilution was shown to have a like effect. This is no doubt due to the fact that twofold dilution in most instances does not cause complete hemolysis, whereas a combination of freezing and twofold dilution does. Figs. 1, 2, and 3 indicate that the conductivity of the red cells rises until hemolysis is complete, after which it falls in much the same manner as the serum. Table III indicates the effect of freezing on the conductivity of the corpuscles.

TABLE III.

Type.	Conductivity × 10^5 of corpuscles alone.		Conductivity × 10^5 of corpuscles diluted twofold.	
	Unfrozen.	Frozen.	Unfrozen.	Frozen.
Rabbit................	82	95	160	228
Pig....................	75	117	192	260

SUMMARY.

The effect of dilution on the osmotic pressure and the electrical conductivity of whole blood, blood serum, and corpuscles is recorded.

Twofold dilution of serum causes approximately a 50 per cent decrease in the osmotic pressure of the same. Twofold dilution of erythrocytes of both nucleated and non-nucleated types causes a decrease of 57 to 60 per cent of the original osmotic pressure to occur.

Tables and charts are shown indicating the changes in conductivity of serum, whole blood, and erythrocytes on dilution.

A greater degree of concentration of electrolyte in serum than in the red cells seems to be indicated although the concentration of electrolyte in the liquid phase of the cell may be equal to, if not greater than, that in the serum.

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OSMOTIC PRESSURE OF TISSUE AS DETERMINED BY THE CRYOSCOPIC METHOD.

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It has been shown by Sabbatani (1) that the osmotic pressure of tissue can be determined by the simple expedient of packing the tissue about the bulb of the Beckmann thermometer contained in the usual form of freezing tube. The tissue, after it has supercooled to a certain degree, suddenly freezes, and the highest point to which the mercury rises in the capillary tube of the thermometer is read off, the Δ thus being determined as in the case of liquids. This method has been applied by Moore (2) in a study of the effects of fatigue on the osmotic pressure of the muscle tissue of the frog.

The constant deviation of the osmotic pressure of the erythrocytes from that of the serum in various vertebrate types observed by the author, and dealt with in detail in Paper I, is suggestive that such differences in osmotic tension might be met with elsewhere, such as between blood and tissue fluid or lymph, or between tissue fluid and the cytoplasm of the cellular elements of the various tissues. The results of the experiments which are reported in this paper suggest that there are actual differences in the osmotic tension of the various tissues, and that of the blood and lymph, and also that there may be an actual difference in osmotic tension between one tissue and another.

The Δ for lymph is as a rule practically the same as that for serum, but slightly higher than that for defibrinated blood. One may therefore assume that the osmotic pressure of the tissue fluid which bathes the cells is approximately the same as that of the blood.

The material studied by the cryoscopic method in this investigation consisted of various tissues and the whole blood of the cat and dog. The blood was aspirated from a large vein while the animal was under ether anesthesia, and defibrinated by shaking with glass beads in an open flask. The tissues were taken when possible while the animal was anesthetized, but failing this they were removed as speedily as possible immediately after death. The tissues were passed at once through a meat grinder, and from 20 to 30 gm. of the minced tissue transferred to the freezing tube, and the Δ was determined immediately. The results of four experiments are given in Table I.

TABLE I.

Tissue.	Experiment 1. Dog. Δ	Experiment 2. Dog. Δ	Experiment 3. Cat. Δ	Experiment 4. Dog. Δ
	°*C.*	°*C.*	°*C.*	°*C.*
Whole blood	0.61	0.64	0.63	0.82
Lymph	0.63			
Heart	0.77	0.81	0.86	
Lung	0.77	0.74		0.86
Spleen	0.77	0.75		0.87
Liver				0.91
Muscle (thigh, abductors)	0.84	0.85		0.94
Brain		0.76		

It will be noted that in all instances where the Δ for a tissue has been determined it is invariably higher than that for defibrinated blood, the difference being very marked in each case.

In Experiments 1, 2, and 3, the animals were under ether anesthesia, while in Experiment 4 the animal was placed under ether anesthesia, then 20 per cent ethyl alcohol in 0.9 per cent sodium chloride was given intravenously, and the ether discontinued. The samples were taken after a period of 5 hours of alcohol anesthesia. The high Δ recorded for whole blood was due in this instance to the alcohol held in solution in the blood.

It is freely admitted that the Δ for tissue determined in the manner outlined above may not be an absolute index of the osmotic pressure of the same in the intact animal. The difference between the Δ of tissue and that of blood is such, however, that a higher osmotic pressure within the tissue cell than without would seem probable. This it may be noted is the reverse condition to that found to exist between the red blood cells and the serum.

It is possible also that a certain portion of the molecular and ionic constituents of the tissue which effect the depression of the freezing point of the same are inactive osmotically within the living tissue, being held in restraint in the form of adsorption layers at the interfaces as suggested by Macallum (3).

Another point which has to be considered is the actual volume of fluid in the tissue cell. It is this which must be in osmotic equilibrium with the tissue fluid without.

It has been shown in Paper II that twofold dilution of red cells causes a decrease of from 57 to 60 per cent in the osmotic pressure while in serum diluted twofold the Δ is lowered 50 per cent. It has been found that tissue reacts similarly to the erythrocytes on dilution as the results reported in Table II indicate.

TABLE II.

Experiment 1. Cat.	Δ	Experiment 2. Dog.	Δ
	°C.		°C.
Muscle	0.98	Heart	0.74
" diluted twofold	0.39	" diluted twofold	0.31

This is a decrease in the Δ as a result of twofold dilution with distilled water, of 60 per cent in Experiment 1 and of 58 per cent in Experiment 2. The application of the arbitrary formula suggested in Paper II $\Delta_1 = \Delta_2 \frac{(100+x)}{x}$ gives in the first instance a value of 66 per cent for actual fluid in the tissue, and 72 per cent in the second instance. If the osmotic tension of the fluid within the cell is approximately the same as the tissue fluid or lymph without, it follows that the quantity of electrolytes held in the tissue, but removed from the sphere of osmotic activity, must be considerable. It has been pointed out by Moore and Roaf (4) that osmotic equilibrium between two media does not necessarily imply that the osmotic tension of each is identical. It is quite possible therefore that the osmotic tension within the tissue cell is greater than without, and at the same time a condition of osmotic equilibrium may exist between the cytoplasm of the cellular elements of the tissue and the tissue fluid. The Δ for tissue is higher than that for blood, due chiefly to the fact that there is more non-electrolyte in the former than in the latter. This is illustrated by the following experiment.

A dog was placed under ether anesthesia, and a sample of venous blood taken; this was defibrinated at once. The thorax was then opened, and the heart excised while still beating. The heart freed of blood, as far as possible, was at once frozen solid by placing in a −20°C. atmosphere out of doors. The frozen heart was then allowed to thaw. It was finally passed through a meat chopper, and the minced tissue was weighed, and mixed thoroughly with four times its weight of distilled water. The mixture was divided into two portions A and B. The Δ of an aliquot of Sample A was then determined, while the remainder of this specimen was allowed to stand in a cool place for 2 hours. It was then centrifuged, the supernatant fluid pipetted off, and its electrical conductivity determined at 37°C. by the Kohlrausch method. Specimen B was autoclaved at 117°C. for a period of 30 minutes. It was then centrifuged and the Δ and electrical conductivity of the clear supernatant fluid were determined.

The specimen of defibrinated blood was diluted with four times its weight of distilled water, and then autoclaved for 30 minutes as in the case of Specimen B of heart tissue. The Δ and electrical conductivity of the supernatant fluid obtained after centrifuging were also taken. The results are shown in Table III.

TABLE III.

	Sample A.	Sample B.	Blood.
Δ heart muscle and blood, *°C*.....	0.158	0.159	0.142
Δ " " " " specific conductivity × 10^5	343	363	425

It will be noticed that autoclaving did not produce an appreciable difference in the Δ while the specific conductivity of the clear fluid obtained on centrifuging was definitely increased. This latter effect has probably a double cause due to the removal of the bulk of the colloidal material as a result of coagulation of the protein, and the more thorough extraction of the tissue. This increase in the specific conductivity while definite is, however, not relatively very great. Such wide differences as regards electrical conductivity of extracts between tissue and blood as are noted in the last experiment quoted were not always manifested. The specific conductivity of the blood extract was, however, in all cases higher than that of tissue extract, while the Δ for the former was less than that of the latter, as the results of a typical experiment indicate (Table IV).

TABLE IV.

Dog under Ether.

Extract plus four times weight of distilled water.	Δ	Specific conductivity × 10^5.
	°C.	
Blood	0.148	456
Pancreas	0.188	395
Muscle (thigh, abductors)	0.177	386
Spleen	0.185	411
Kidney	0.188	425
Heart	0.183	427

The fact that the Δ for tissue is higher than that for blood and also that the Δ for tissue mixed with a definite amount of distilled water is greater than that for blood similarly treated, indicates a higher concentration of total solutes in the tissue than in the blood. On the other hand the lower specific conductivity of tissue extracts, made as described above, compared with blood extracts so made, probably indicates that the free electrolyte in the tissue is not in excess of that of the blood, or that it may be even less in amount. A further experiment, the results of which indicate that the total solute concentration in the tissue is higher than that of the blood, is quoted.

The heart of a cat was excised, weighed, minced, and mixed with four volumes of distilled water. The mixture was autoclaved for 30 minutes at 117°C. It was then filtered, and an aliquot volume of the filtrate reduced on the water bath to an aliquot weight of the original tissue. The Δ for this was then taken; it was 0.75°C., a reading much higher than is ever given by normal cat blood.

It may be urged that in all the experiments quoted sufficient autolysis might occur during the period which elapses between the time of sampling and the actual recording of the depression of the freezing point to account for the difference between the Δ of tissue and that of blood. While there can be no doubt that a slight increase in the Δ would be caused by autolysis, yet it is the opinion of the author that the differences observed in the Δ for tissues and that for blood are such as to indicate that a definite difference in actual solute concentration does exist between the cytoplasm of the tissue cells and the blood or lymph. The presence of amino-acids in the tissue in much greater concentration than in the blood (5) would account in part at least for this difference in concentration with regard to the total solutes.

SUMMARY.

The Δ for tissue is higher than that for blood or lymph.

The specific conductivity of tissue extracts is lower than that for the blood extracts similarly prepared.

The results recorded point to a higher osmotic tension within the tissue cell than in the blood or lymph.

The concentration of non-electrolyte is greater in the tissue cell than in the blood or lymph.

The concentration of electrolyte is probably greater in the blood than in the tissue.

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MAINTENANCE OF OSMOTIC PRESSURE WITHIN THE NUCLEUS.

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Plates 1 to 3.

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It is generally understood that there is a state of osmotic equilibrium existing between the blood plasma and the tissue fluid from which it is separated by the endothelial wall of the capillary, and between the tissue fluid and the cytoplasm of the tissue cells which are separated by the limiting layer of the individual cells. It is also apparent that a condition of osmotic equilibrium must exist between the cytoplasm of the tissue cells and their respective nuclei. It has long been known that the amount of any one element or salt may vary in different tissues. We may take for example the difference in concentration of sodium and potassium in the serum and in the red cell. Microchemical investigations by Macallum (1) have shown that there is a definite distribution of certain ions in each tissue, and the type of distribution of one particular ion in one tissue is not necessarily the same as that in another. Moore, Roaf and Webster (2) have attributed the specific differences, both quantitative and qualitative, in the electrolyte content of different tissues to special affinities between colloids of various types and electrolytes of various types.

It has been found by the author in a microchemical study of various tissues, following the methods evolved by Macallum (1), that the nucleus is free from potassium, chlorine, phosphate, and carbonate ions, thus confirming the observations of Macallum. Results such as these suggest one of two things: either the nucleus is electrolyte-free, or the technique followed in the course of such microchemical investigations is not sufficiently perfect to demonstrate electrolyte in all instances where it actually occurs.

The tissue to be studied was removed from the animal while under ether anesthesia or else cut away as quickly as possible after the animal had been stunned by a blow on the head. A small block of the tissue was then transferred to the chilled stage of a freezing microtome which was inclosed in an asbestos-lined box fitted with a plate glass cover. The process of freezing was almost instantaneous. Distilled water was used to make an ice embedding about the block of tissue. Ten micron sections were cut, a chilled knife being used. The sections were taken up on camel's hair brushes, previously moistened with distilled water and subsequently frozen, and dropped while still frozen into the reagent which was contained in a porcelain basin within the freezing chamber. The air within the freezing chamber was kept well chilled by means of an additional carbon dioxide spray. This latter was also used for chilling the sectioning knife.

When this technique is followed one obtains very clear-cut localizations of potassium, chlorine, phosphate, and carbonate, such as have been described by Macallum (1) and the nuclei throughout appear to be free of electrolyte.

The evidence such as is furnished by microchemical investigation points to the absence of the common inorganic electrolytes from the nucleus. It may be suggested that failure to obtain a reaction in the nucleus for ions which have been localized elsewhere in the tissue may be due to non-penetration of the reagent into the nucleus on account of impermeability of the nuclear membrane. The answer to such a criticism would be that in the method of sectioning outlined above one is certain to cut through nuclei on several occasions and in such cases the permeability of the nuclear membrane to the precipitating reagent, such as cobalt sodium hexanitrite, or silver nitrate, could not be considered as a factor modifying any nuclear reaction which might otherwise occur.

The writer is therefore of the opinion that the results of microchemical investigations carried out as outlined above are adequate proof of the existence in the nucleus of only the slightest traces of inorganic electrolyte. This would seem to be a condition of marked significance, for, if the nuclear fluid is in osmotic equilibrium with the cytoplasmic fluid and therefore has an osmotic tension approximate at least with that of the latter, this tension must be maintained for the most part by non-electrolyte or organic electrolyte. It would follow from this that, since the concentration of electrolyte in the nucleus is minimal, concentration of non-electrolyte must here be maximal.

It has been shown in Paper III of this series that the concentration of free electrolyte in tissue is probably slightly less than in blood serum, but it is nevertheless true that the osmotic tension of the tissue as a whole is largely determined by the free electrolyte contained in the same. The nucleus, however, if electrolyte-free, must contain non-electrolyte in concentration equivalent to that of an approximately $\frac{1}{3}$ molar solution.

A number of photomicrographs and drawings of tissue sections which have been treated according to the technique previously outlined, with a view to localizing potassium, chloride, phosphate, and carbonate ions, are shown in Figs. 1 to 14. The nuclei of the tissue cells are in practically every instance conspicuous, due to the almost total absence of any reaction in them.

It has been shown by Macallum and Collip (3) that there is in ganglionic nerve cells a substance (probably an hydroxy-benzene derivative) which will reduce silver nitrate in neutral solution. This substance is confined to the cytoplasm of the nerve cells of vertebrate types studied, while in the nerve cells of the leech it appears to extend out into the axon for a short distance (Fig. 1). The entire absence of any reaction with nitrate of silver in neutral solution within the nucleus is the point to be emphasized. This is shown quite distinctly in Fig. 2. The tissue in this instance is a terminal sympathetic ganglion adjacent to the cortical substance of the suprarenal gland. Camera lucida drawings (Figs. 3, 4, and 5) of ganglionic nerve cells which had been treated with silver nitrate in neutral solution also illustrate the absence of any nuclear reaction. Fig. 6 illustrates the distribution of chlorides in ganglionic nerve cell and fiber. It has been shown (4) that chlorides are present in the parietal cells of the gastric tubules in considerable quantity during the stage of active secretion, while during the resting stage they are present in these cells in traces only. In either phase, however, the nucleus is chloride-free. It was also demonstrated that phosphates and carbonates are at all times present in the parietal cells, irrespective of whether they are in the active or resting stage. Figs. 7 and 8 illustrate the distribution of chlorides in the active gastric tubule of the dog.

The luminal half of the cells lining the tubules of the proventriculus of the hen was found to show changes somewhat similar to those noted in the parietal cells of the mammalian stomach in regard to chloride content during the resting and active stages, but here the localization is much more clearly defined, both as regards chlorides, and phosphates and carbonates. Figs. 9, 10, and 11 illustrate the peculiar type of localization. The nucleus is free from any reaction.

At no time were the nuclei of any tissues studied found to be impregnated with ions of potassium, chlorine, phosphate, or carbonate so as to give an appreciable reaction.

It has been demonstrated by Macallum (5) that the cytoplasm as well as the nucleus of nerve cells is potassium-free. Fig. 12 illustrates the distribution of potassium in the Gasserian ganglion of the dog. Brown (6) has shown that the nuclei of the cells of the renal tubule are potassium-free while Defries (7) found no potassium in the nuclei of the salivary or pancreatic gland cells. Macallum (1) has found the nucleus free from all inorganic salt in tissues studied by him.

It is, of course, impossible by microchemical methods to prove absolutely the absence of free inorganic electrolyte from the nucleus. The fact, however, that the ions of chlorine, phosphate, and carbonate are uniformly absent or present in traces only would point to the absence of balancing cations such as those of sodium, potassium, calcium, and magnesium. The absence of potassium from the nucleus is all the absolute data which is yet available as regards its cation content.

The data which have been presented would seem sufficient justification for the theory that the nucleus is very low in total inorganic electrolyte content if not entirely electrolyte-free.

Assuming that such is the case, what evidence is there at hand which will indicate the means by which the osmotic tension of the nucleus is maintained?

The non-electrolytes or organic electrolytes which might possibly occur in greater concentration within the nucleus than in the cytoplasm, tissue fluid, or blood plasma are dextrose, urea, ammonium salts, organic acids (chief among which would be free amino-acids), and other organic substances of low molecular weight.

A certain amount of useful data would be furnished by making quantitative analyses for these various substances in the various tissues. Tissues in which the nuclei are large and make up an appreciable part of the total tissue bulk could be compared with tissues in which cytoplasm is far in excess of the nuclear elements.

It has been found, using the Greenwald method (8), that the total non-protein nitrogen of avian blood is from three to five times as concentrated in the red cells as in the plasma, whereas in mammalian blood the differences noted are of an absolutely different type. Similarly using the Van Slyke and Meyer alcohol method (9) for estimating amino-acid nitrogen, it was found that the nitrogen gas evolved in 3½ minutes at 22°C. from the alcohol-free extract of plasma and erythrocytes of avian blood, calculated as amino-acid nitrogen, gave a value from three to five times as great for the erythrocytes as the plasma, whereas in mammalian blood the amino nitrogen in the corpuscles was approximately the same as in the plasma. The results of these analyses are shown in Table I.

Constantino (10), using the formal method of Sörensen, found that mammalian serum and corpuscles during fasting are constant in their amino-acid nitrogen. He found 4.4 mg. of amino-acid nitrogen per 100 gm. of dog serum and 3.2 mg. per 100 gm. of ox corpuscles. Van Slyke and Meyer (9), using the alcohol precipitation method, found 3 to 5 mg. of amino-acid nitrogen per 100 cc. of blood of dogs which had been fasting 20 to 24 hours. Constantino (10) reports finding 10 mg. of amino-acid nitrogen in 100 gm. of blood obtained from dogs during full digestion. Bock (11), using his own method, found an average of 7.47 mg. of amino-acid nitrogen per 100 cc. of dog blood and 8.43 mg. per 100 cc. of pig blood. Constantino (10) found 3 mg. of amino-acid nitrogen in 100 gm. of turkey blood and 34 mg. in 100 gm. of corpuscles. Bock (12) reports the amino-acid content of bird blood as roughly three times as high as that of mammals. Thus he found the whole blood of the chicken to contain 23.79 mg. of amino nitrogen per 100 cc. of blood divided as follows: 7.79 mg. in the plasma and 16 mg. in the cells. Bock (11) found 20.99 mg. of amino nitrogen as the average for 100 cc. of chicken blood, 21.32 mg. per 100 cc. of duck blood, 20 mg. per 100 cc. of turkey blood, and 18.6 mg. per 100 cc. of goose blood. Wilson and Adolph (13) found the amino nitrogen of whole blood of fish to vary from 17 to 34 mg. while that in the plasma varied from 7 to 18 mg.

In my own analyses the blood was oxalated as it was drawn. It was then centrifuged and the plasma and corpuscles were at once pipetted off into separate graduates. The results reported in

Table I may be a little low as, according to Bock (12), the alcohol precipitation method of Van Slyke and Meyer (9) is not absolutely reliable due to adsorption of some of the amino-acids by the precipitated protein. The urea was not removed in the cases here reported nor was any calculation made to determine the amount of this constituent.

TABLE I.

	Amino-acid N per 100 cc.		Total non-protein N per 100 cc.	
	mg.	*mg.*	*mg.*	*mg.*
Hen cells	37	45	126	
" serum	9	7	35	
Duck cells	34		128	
" serum	6.5		24	
Pigeon cells	36		125	
" serum	11.5		35	
Turkey cells	36	33*	133	
" serum	6	10*	26	
Sheep cells	5		35	
" serum	5		23	
Dog cells			45	
" serum			31	
Goose cells	35*	35*	135*	120*
" serum	11	12	42	29

*In full digestion.

When the actual bulk of the nucleus is taken into consideration there is sufficient non-electrolyte or organic electrolyte (assuming the excess of total non-protein nitrogen in the nucleated red cells over that in the corresponding plasma to be largely confined to the nucleus) within the nucleus to maintain an osmotic tension equal to that of the surrounding cytoplasm where the osmotic tension is, in large measure, due to inorganic electrolyte.

That there is a definite nuclear membrane, the permeability of which must enter into any consideration of exchanges between nucleus and cytoplasm, is well established. The author has been successful in dissecting nuclei free from nerve cells and photographing the same while under the ultramicroscope. Fig. 13 shows two such nuclei, the cytoplasm of which has been torn away, in the same field with a damaged nerve cell, the nucleus of which is still intact. The source of these nerve cells was a ventral ganglion of the medicinal leech. The absolute homogeneity of the nucleus as viewed under the ultramicroscope stands in sharp comparison with the heterogeneity of the cytoplasm. Fig. 14 shows two normal nerve cells as photographed under the ultramicroscope.

One finds in the case of tissue other than blood that the concentration of total non-protein nitrogen is far in excess of that in the blood and that the same is true of amino-acid. Van Slyke and Meyer (14) have found that the concentration of amino-acid in the tissue is normally from five to ten times that in the blood. They hold that the process by which amino-acids are taken up and held by tissue cannot be wholly osmotic and suggest either the possibility of mechanical adsorption of the amino-acid by the tissue or the formation of loose molecular compounds between the amino-acids and the tissue proteins.

While there is no absolute justification for applying the conclusions reached as regards the maintenance of the osmotic tension of the nucleus of avian blood cells to nuclei of tissue generally, yet it would appear to the writer, in the light of microchemical studies and of quantitative analyses of tissue, that in the nuclei of any tissue there is a higher concentration of total non-protein nitrogen and also of amino-acid than in the cytoplasm, the tissue fluid, or the blood. If the cell membrane is freely permeable to amino-acid, it is difficult to understand why there should be such a marked difference in the content of amino-acid in the blood and tissue. If, however, a large percentage of the tissue amino-acid could be shown to be confined to the nuclei, then one would not only be able to explain the difference in concentration in the amount of this substance in the blood and tissue but one would add an additional function, as well as significance, to the free amino-acid of the tissue; namely, that of acting in a purely physical manner, helping to maintain the osmotic tension of the nucleus.

If the intact nucleus is free of inorganic salts, it would be of great interest to know whether electrolyte gets in among the chromosomes during the stage of mitosis. The writer hopes to have some information on this point shortly. It may be stated here, however, that chlorides, phosphates, and carbonates are absent from the entire cell in the case of the sprouting root tips of the onion where mitotic figures are very much in evidence. The writer is not, however, overconfident of these results, as the usual method of sectioning could not be applied in this instance and the possibility of non-penetration of the silver nitrate reagent into the cell was not disposed of in this experiment. The results are nevertheless suggestive.

SUMMARY.

The freedom of the nucleus from inorganic electrolyte such as chloride, phosphate, and carbonate of potassium is demonstrated.

That there is not only a condition of osmotic equilibrium existing between the blood plasma and the tissue fluid, and the tissue fluid and the cytoplasm of the tissue cells, but also an osmotic equilibrium between cytoplasm and the nucleus in each nucleated cell is pointed out.

As the osmotic tension of the nucleus cannot be maintained by inorganic electrolyte, it is argued that non-electrolyte or organic electrolyte must account for the nuclear osmotic tension.

Evidence is given to show that the concentration of total non-protein nitrogen within the nucleated erythrocytes is from three to five times as great as the concentration in the corresponding plasma.

Evidence is quoted and further evidence given to show that the concentration of amino nitrogen is three to five times as great in the nucleated erythrocytes as in the corresponding serum.

It is assumed that the excess of amino-acid and other nitrogenous solutes of small molecular weight in the cells of nucleated types of blood over that in the plasma is largely confined to the nucleus.

It is suggested that the concentration of these substances as indicated in the nucleus is sufficient to maintain the osmotic tension of the same.

It is held that the conclusions reached as regards the maintenance of the osmotic tension of the nuclei of the avian erythrocytes are generally applicable to nuclei of tissue.

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EXPLANATION OF PLATES.

Figs. 1, 2, 9, 10, 12, 13, and 14 are photomicrographs. Figs. 3, 4, 5, 6, 7, 8, and 11 are drawings.

PLATE 1.

FIG. 1. Reaction in ventral ganglion of *Hirudo medicinalis* treated with 0.1 N silver nitrate in neutral solution.

FIG. 2. Reaction in nerve cell of sympathetic ganglion attached to cortex of suprarenal gland, treated with 0.1 N silver nitrate in neutral solution. Dog.

FIGS. 3, 4, AND 5. Reaction in ganglionic cells, treated with 0.1 N silver nitrate solution. Dog.

FIG. 6. Localization of chlorides in nerve cell and axones of Gasserian ganglion. Dog.

PLATE 2.

FIG. 7. Localization of chlorides in the active gastric tubule. Dog.

FIG. 8. Localization of chlorides, phosphates, and carbonates in the active gastric tubule. Dog.

FIG. 9. Localization of chlorides, phosphates, and carbonates in the active proventriculus. Fowl.

FIG. 10. Localization of chlorides, phosphates, and carbonates in the resting proventriculus. Fowl.

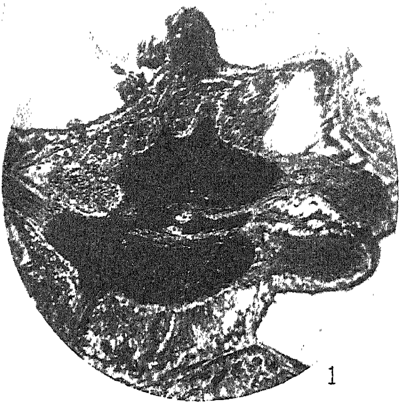
FIG. 11. Localization of chlorides, phosphates, and carbonates in the active proventriculus. Fowl.

PLATE 3.

FIG. 12. Localization of potassium about nerve cells of Gasserian ganglion. Dog.

FIG. 13. Two nuclei and one damaged nerve cell, photographed under ultramicroscope. *Hirudo medicinalis.*

FIG. 14. Two normal nerve cells, photographed under ultramicroscope. *Hirudo medicinalis.*



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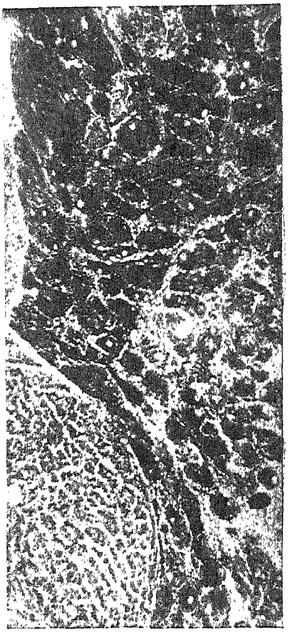

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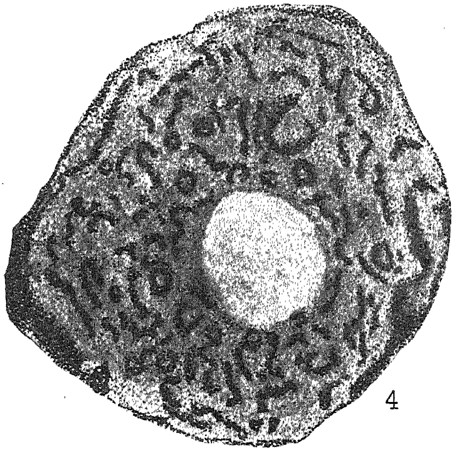

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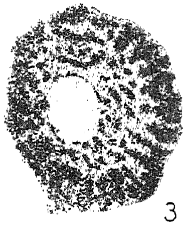

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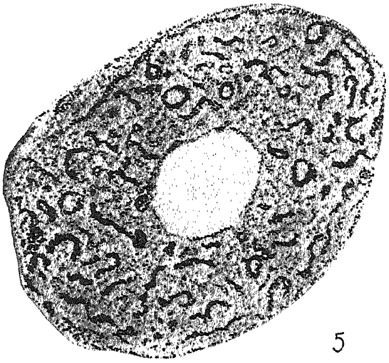

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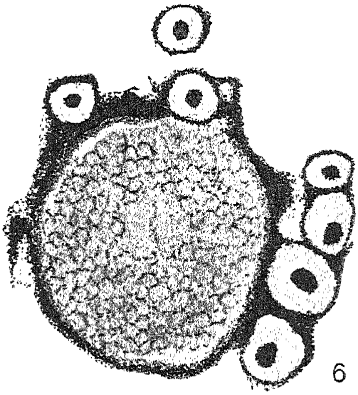

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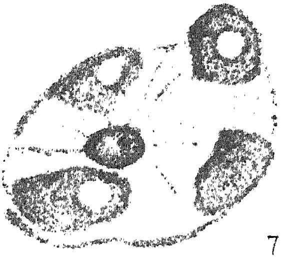
(Collip: Osmosis. IV.)



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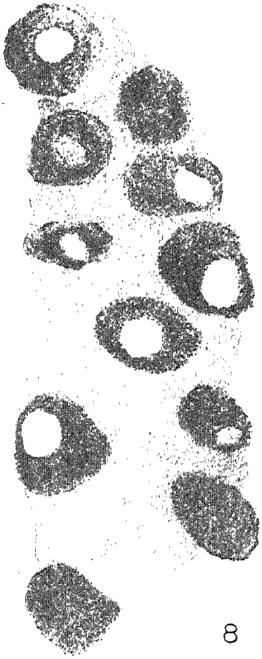

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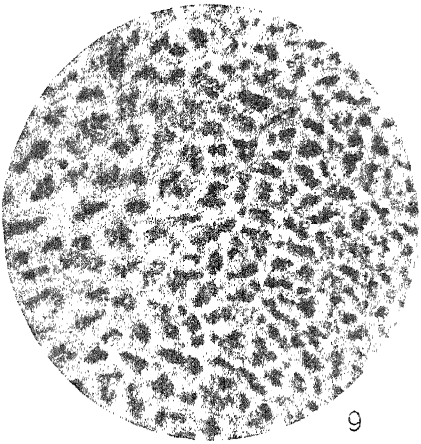

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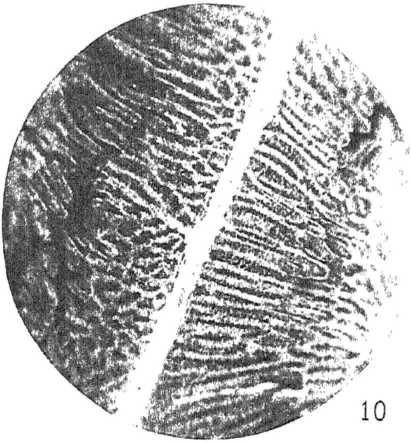

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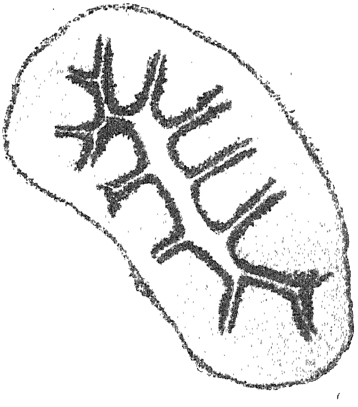

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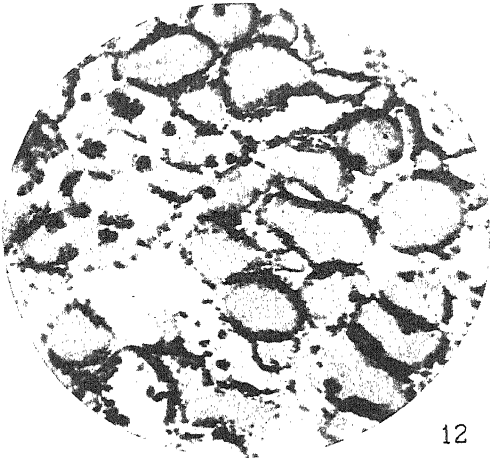

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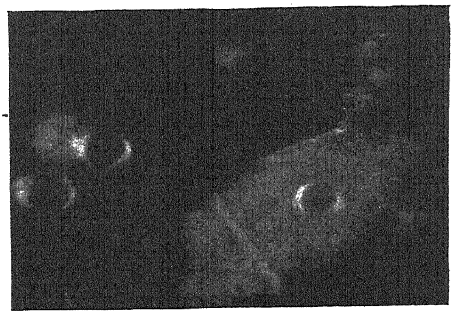

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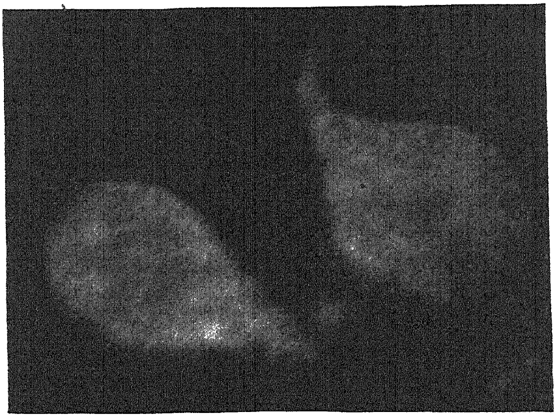

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(Collip: Osmosis. IV.)

HEMATO-RESPIRATORY FUNCTIONS.*

II. THE HENDERSON-MORRISS METHOD FOR DETERMINING THE CARBON DIOXIDE IN PLASMA AND IN WHOLE BLOOD.

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(Received for publication, April 19, 1920.)

A subnormal CO_2-combining power in the plasma of the blood is now regarded as an index of acidosis. The values obtained from whole blood have to a large extent been neglected. For reasons which will be stated in a succeeding paper it is often important and sometimes essential to use the whole blood. Whole blood has also the advantage of economy; to get a unit of plasma one needs two or three units of blood.

The use of the CO_2-combining power as an index of the alkali and thus indirectly of the C_H of the blood, was introduced by Hasselbalch and developed by Van Slyke. Hasselbalch regards determinations on plasma as much less significant than on whole blood. The former may at times be absolutely misleading. After careful comparison of whole blood and plasma Hasselbalch and Warburg (1) concluded that the CO_2-binding power of a serum rises and falls with the CO_2 tension of the blood from which it is separated. It is not possible to draw from the CO_2 of the serum any exact conclusion whatever regarding the blood reaction, unless the CO_2 tension of the blood, when the serum was separated, has been taken into account. Experience in this laboratory fully confirms this statement. In fact, although Van Slyke's statement to a similar effect has been generally overlooked, the truth of this was emphasized by him also. Carbonating the plasma after separation is comparatively unimportant.

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* This work was done under the War Gas Investigations of the Bureau of Mines and the Chemical Warfare Service, U. S. Army. The writer is indebted to H. H. Beatty, S. R. Detwiler, and W. H. Taliaferro for valuable collaboration.

In a paper from this laboratory (2) published a little over 2 years ago a simple method was described for determining the CO_2 content and capacity, or combining power, of the whole blood. Since that time this method has been in continuous use on an extensive scale in this laboratory, and we have now acquired an experience which allows us to judge of its value.

The Van Slyke and Cullen (3) method for determining the CO_2 capacity, or alkaline reserve, of plasma has been used by us interchangeably with this method. For plasma the two methods are of about equal rapidity, simplicity, and accuracy. For the determination of CO_2 in whole blood the necessity of cleaning the Van Slyke apparatus after each analysis is troublesome and time-consuming. The Henderson-Morriss method offers no more difficulties with whole blood than with plasma. The gas burette serves also to analyze the equilibrating air—an essential for exact work.

The paper describing the Henderson-Morriss method was of a preliminary character. It was therefore suggested to us by Henderson that we reconsider in detail the calculation of results by this method and report a series of carefully performed comparative determinations with the Van Slyke procedure; also, that we rectify a typographical error in the barometric correction.[1]

In the Henderson-Morriss method 1 cc. of blood is laked with dilute ammonia and then acidulated with tartaric acid, 20 per cent, in a glass tube of simple form which contains when stoppered exactly 3.5 cc. of acid-blood mixture and approximately 24 cc. of air. The tube is then rolled so that the fluid spreads in a thin film allowing free diffusion between fluid and air. 3 minutes are sufficient to bring the tension of the CO_2 in the air and fluid to equilibrium. The tube is then connected with the gas burette. The lower end is in a pan of water. The stopper is withdrawn under water. The total amount of gas (air plus the CO_2 liberated by acid) is drawn into the burette, water following up the tube from the pan. The end of the tube is lifted sufficiently to admit a bubble of air which is then used to wash the last portion of the air in the tube into the burette. The burette is then read, the gas passed five times into the absorbing bottle containing 10 per cent NaOH, and the burette is again read. The decrease of volume observed, when subjected to the corrections described below, indicates the volumes per cent of CO_2 contained in the blood. With a burette of this type, time must be allowed for the fluid, acidulated water, to drain fully before each reading. Quicker draining without loss of accuracy in any other respect is afforded if water plus 10 per cent alcohol and a few drops of HCl are used in the burette. It is well also to add a few drops of litmus, phenolphthalein, or other indicator to this fluid so as to warn one in case any of the alkali is drawn over by mistake.

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[1] Henderson and Morriss (2), p. 224.

Our practice is to measure the blood in a Mohr pipette which has been recalibrated with mercury. The outside of the pipette should be carefully wiped free from blood before the contents are delivered into the diffusion tube. The commonest source of error is in a "long" measurement with the pipette.

We have found it advisable to substitute a 2-way stop-cock for the pinch-clamp between the burette and the absorption bottle as originally figured.

A correction is made for the CO_2 liberated, on acidulation, from the small traces of carbonate in the ammonia solution. This covers also the atmospheric CO_2 in the air in the diffusion tube. The correction consists in subtracting from the observed volume of CO_2 as read on the burette the value obtained from a blank determination in which distilled water replaces the blood.

The observed reduction of volume in the gas burette, *i.e.* the CO_2 absorbed by the alkali, is reduced to the volume of the dry gas at 760 mm. by the formula

$$V_1 = \left(\frac{B - p}{760}\right)(V - \text{Cor.})$$

$(V - \text{Cor.})$ = Volume of CO_2 after correction for carbonate in reagents.
p = Vapor tension of water vapor at temperature of analysis.
B = Barometer reading.
V_1 = Volume of dry gas at 760 mm.

To the volume of dry gas is added the CO_2 retained in solution in the fluid in the diffusion tube. This is estimated by the formula

$$V_2 = V_1 + V_1\left(\frac{3.5}{24} \times c\right)$$

V_2 = Volume of CO_2 in the gas analyzed + that remaining in solution in the acidulated blood.
V_1 = As above.
3.5 = Fluid contained in diffusion tube.
24 = Air " " " "
c = Solubility coefficient of CO_2 in water at temperature of analysis.

The correctness of this value for c as applied to the acid-blood mixture will be discussed later.

The value of V_2 at the temperature of analysis is brought to 0° by the formula

$$V_3 = V_2\left(\frac{273}{273+t}\right)$$

V_3 = Volume at 0°.
V_2 = As above.
t = Temperature of analysis.

The volume of CO_2 in cc. at 0° (*i.e.*, V_3) multiplied by 100 gives the volumes per cent of CO_2 in the blood.

The Solubility Coefficient of CO_2 in the Acid-Blood Mixture.

The solubility coefficient of CO_2 in the acidulated blood is so nearly identical with that for CO_2 in water that the established values for the latter may be used in determining the amount of CO_2 left in solution after the equilibrating air has been analyzed. This was determined by displacing the gas with mercury instead of water (so as not to expose or dilute the acidulated blood), then drawing air freed from CO_2 into the diffusion tube, and again equilibrating. From the analyses of the gas thus obtained in a series of equilibrations amounts of CO_2 were found by analysis approaching zero as a limit. The sum of these added to the CO_2 of the initial equilibration gives the total volume of CO_2 in the blood. For these analyses a gas burette more finely graduated than that generally employed was used. The results of such determinations are given in Table I.

TABLE I.

CO_2 Retained in Acid-Blood Mixture.

Barometer 758 mm.

Blood samples.	Temperature.	CO_2 in equilibration.					
		No. 1.	No. 2.	No. 3.	No. 4.	Sum of Nos. 2, 3, and 4.	Theoretical for distilled water.
	°*C*.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
A	21	0.34	0.040	0.005	0.000	0.045	0.044
B	21	0.53	0.065	0.010	0.005	0.080	0.069
C	21	0.47	0.065	0.010	0.000	0.075	0.061
D	18	0.44	0.060	0.005	0.005	0.070	0.062
E	18	0.38	0.050	0.005	0.005	0.060	0.054

From these figures we conclude that the CO_2 retained in solution in the fluid in the diffusion tube can be calculated by the employment of the solubility coefficient of water to within 1 volume per cent.

The various corrections and calculations just described when applied to this method so neutralize each other that for routine work they are unessential. *The volume of CO_2 absorbed from the air in the burette (minus only the correction for CO_2 in the ammonia* *solution and room air) at ordinary barometric pressure (750 to 770 mm.) and room temperature between 18–25° is identical within the error of the method (1 or 2 volumes per cent) with the figure obtained after making all corrections.*

By subtracting the CO_2 in simple solution in the original blood (4) (3 volumes per cent at normal arterial and pulmonary CO_2 tension and temperature) a figure for the combined CO_2, or alkaline reserve, is obtained.

TABLE II.

Analysis of Sodium Carbonate Solution.

A solution of sodium carbonate from the dried recrystallized salt was prepared by weight. This was diluted as desired and 1 cc. of suitable concentration delivered into the diffusion tube and analyzed as for blood.

Temperature 22°, barometer 757 mm.

CO_2 as read from burette.	CO_2 after correction.	Theoretical CO_2.	Difference.
cc.	*vol. per cent*	*vol. per cent*	*vol. per cent*
1.08	109.0	111.0	−2
1.09	110.0	111.0	−1
0.90	90.0	89.0	+1
0.89	89.0	89.0	0
0.66	66.0	67.0	−1
0.66	66.0	67.0	−1
0.44	44.0	45.0	−1
0.45	45.0	45.0	0
0.21	20.0	22.0	−2
0.23	21.0	22.0	−1
0.12	12.0	11.0	+1
0.10	10.0	11.0	−1
0.01	1.0	0.0	+1
0.00	0.0	0.0	0

As a check on the accuracy of this method we have analyzed sodium carbonate and find that an accuracy within 2 volumes per cent of the theoretical is attainable. The results of a series of such analyses are given in Table II.

In many experiments we have employed the Van Slyke method as a check on that of Henderson and *vice versa.* The comparative results obtained are illustrated by the following figures, in which the first in each case is that by the former, the second that by the latter method.

Plasma: 53, 52; 57, 57; 61, 62; 54, 54; 59, 59; 71, 70; 62, 62; 37, 36; 35, 35; 30, 29; 22, 22; 17, 18; 7, 9; 6, 8; 19, 19; 19, 18; 14, 16; 59, 58; 55, 55; 52, 52; 44, 44; 44, 43; 43, 43; 32, 30; 27, 28; 17, 18; 20, 20; 22, 21; 19, 20; 23, 23; 18, 18; 23, 23; 21, 23; 62, 62; 59, 60; 49, 49; 33, 32; 47, 47; 51, 50; 39, 38.

Whole blood: 44, 46; 43, 42; 49, 47; 37, 37; 49, 49; 32, 32; 27, 29; 27, 30; 31, 31; 42, 44.

The CO_2 Content of Whole Blood.

The CO_2 content is determined by direct analysis of blood drawn from vein or artery without loss of CO_2. If the blood is collected rapidly under mineral oil and at once pipetted into the diffusion tube, no anticoagulant is necessary. For routine work, however, or where it is desirable to determine the CO_2 capacity, coagulation is prevented by powdered ammonium or sodium oxalate. This must be of absolute purity, and an amount should be used which will bring the total concentration to approximately 1 per cent. The technique for drawing and oxalating blood described by Van Slyke and Cullen (3) gives excellent results.

The CO_2 Capacity of Whole Blood.

The CO_2-combining power or capacity is estimated by determining the CO_2 content after the blood has been equilibrated with a definite tension of CO_2 under standard conditions of temperature and pressure. The tension of CO_2 now generally used for equilibration is that of the normal alveolar air: that is, 40 mm. partial pressure. We find that the employment of the last portion of the operator's expired air is inexact and involves large variations in CO_2 content when applied to whole blood. The air employed is best prepared by a modification of the Higgins-Plesch technique. A deep breath is expired into a bag and reinspired twice. The CO_2 tension of the air so obtained should always be established by analysis. The burette used for the blood determinations lends itself readily to this purpose. To get the tension in mm. the percentage of CO_2 found by analysis is multiplied by the barometer minus the vapor tension of water at the temperature at which the equilibration of the blood with this air is performed.

For the equilibration of whole blood the most satisfactory technique is the following. 5 cc. of blood free from mineral oil are pipetted into a 250 cc. Erlenmeyer flask tipped at an angle. The gas from the bag is passed slowly for 1 minute well beneath the surface of the fluid by means of a glass tube. With the gas still flowing and the flask full of foam, the tube is withdrawn and the stopper at once inserted. The flask is shaken continuously for at least 5 minutes with a rotary motion to spread the blood in a film over the surface and expedite the equilibrium which is complete at the end of 5 minutes.

In performing the saturation at a temperature higher than that of the surrounding air, the flask is immersed in a water bath. The increased pressure is relieved by loosening for a moment the edge of the stopper.

As regards the influence of temperature on the CO_2-combining power, or as we term it "the alkali called into use," in blood we have found that the effect varies in exact proportion to the amount of CO_2 dissolved at different temperatures. In other words, in a given blood put through a variety of temperatures without loss or gain of total CO_2, the tension of CO_2 varies, but the ratio of dissolved to combined CO_2 does not.

CONCLUSIONS.

1. In the Henderson-Morriss method of determining the CO_2 of plasma or whole blood it is found that the various corrections almost exactly neutralize each other. At ordinary temperatures and barometric pressures the reading on the gas burette for the CO_2 absorbed during the gas analysis (corrected only for the carbonate of the ammonia solution) may be taken as indicating directly, within the error of the method, the amount of CO_2 contained in the blood or plasma. Each 0.01 cc. corresponds to 1 volume per cent of CO_2.

2. This method is particularly valuable for the determination of the CO_2 content and combining power of whole blood: With it the analysis of whole blood is as easy as that of plasma.

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EXCHANGE OF CHLORIDE IONS AND OF CARBON DIOXIDE BETWEEN BLOOD CORPUSCLES AND BLOOD PLASMA.

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Hamburger (1891) found that chlorides pass from the blood plasma into the red blood corpuscles when pure carbon dioxide is led through a sample of blood. In Hamburger's experiment, defibrinated blood was shaken with air, and the NaCl in the serum determined. Pure CO_2 was led through a sample of the same defibrinated blood and the amount of the NaCl in the serum fell from 0.58 to 0.53 per cent, 0.05 per cent having passed into the red corpuscles. The change proved to be reversible, the percentage of NaCl in serum increasing again when the CO_2-treated blood was shaken with air.

The experiment of Hamburger has been repeated several times with variations (Gürber, Koeppe, Petry, von Limbeck, and others). In his first paper Hamburger reported that in addition to chlorides other anions of the serum could pass into the corpuscles under the influence of CO_2, and this has recently been confirmed by new experiments of de Boer and of Hamburger himself (1918).

The experiments on exchange of anions between plasma and corpuscles have been criticized by Overton, who pointed out that in all the experiments the blood corpuscles had been treated with pure CO_2, and this procedure possibly had changed their permeability. Overton thought that uninjured blood corpuscles were impermeable for anions.

The next step is to determine what happens with the plasma chlorides if blood is saturated with different mixtures of carbon dioxide and air, especially whether or not a change in the tension of CO_2 has any effect upon the distribution of the blood chlorides by low CO_2 tensions.

My first experiments deal with this point. In 1917 Van Slyke and Cullen in a paper dealing chiefly with other problems published experiments on the influence of different tensions of CO_2 on the distribution of the blood chlorides between plasma and corpuscles. My results are identical with theirs on some points. Still I shall briefly report my experiments on this point as an introduction to my further experiments and because I have used a different method.

Methods.

All experiments were carried out on fresh ox blood, the clotting being prevented by 0.1 per cent potassium oxalate. The volume of red blood corpuscles was estimated in an improvised hematocrit. The amount of hemoglobin was estimated by a standardized Haldane hemoglobinometer, the results being given in volumes per cent of oxygen capacity.

The saturation of blood samples with mixtures of air and carbon dioxide took place in saturaters of glass according to the method of Barcroft. Each saturater had a volume of 275 cc.; 3 cc. of blood were used for each experiment. One end of the saturater was closed by a stopper with a capillary glass tube through which samples of the air mixture in the saturater could be taken when the saturation of the blood was finished. The other end of the saturater was narrowed into a glass tube of the same diameter as a small centrifuge test-tube, being connected air-tight with this by means of a piece of rubber tube. After the saturation the blood was run into the centrifuge test-tube which was almost filled. After the piece of rubber tube had been pinched the centrifuge tube with the blood and the closed rubber tube as a stopper could be placed in a centrifuge without coming into contact with air other than that of the saturater.

The air sample from the saturater was analyzed in the Haldane apparatus.

The saturation took place by rotating the saturater in a water bath at 14–17°C. As no provisions were made to heat the centrifuge it was necessary to saturate at room temperature.

After centrifugation, samples of the blood plasma were taken for the determination of chlorides and eventually of carbon dioxide.[1]

The estimation of chlorides in the plasma was carried out after the micro method of Bang, not more than 0.1 cc. of blood being required for an estimation. I examined the accuracy of Bang's method in a series of experiments and found that the average error on a single estimation is 1.4 per cent of the total amount of chlorides. It is indifferent whether the amount of blood is measured in a calibrated pipette or weighed on a torsion balance, as Bang himself prefers. If the blood (or plasma) is determined by weight, a correction for loss by evaporation has to be introduced. In my experiments the blood was measured in a pipette. In every experiment I made three estimations of the amount of chlorides in the plasma, which reduces the average error to 0.6 per cent. Therefore variations of more than 2 per cent are real and cannot be ascribed to analytical errors. The second decimal is correct in estimations in blood or plasma (about 0.5 per cent of chlorides being found); the third is quite unreliable, but is given in the experimental accounts. The amounts of chlorides are conventionally given in percentage of NaCl, although this has certain disadvantages.

EXPERIMENTAL.

Exchange of Chloride Ions.

In Experiments 1, 2, and 3 blood was saturated with air containing different amounts of carbon dioxide, the chlorides being estimated in the plasma after the saturation.

Experiment 1.—Temperature 12°C.; Blood: oxygen capacity 18.3 volumes per cent, 0.418 per cent of NaCl.

CO_2 tension, *mm*	5.7	53.0	73.0	79.0
NaCl in plasma, *per cent*	0.528	0.510	0.512	0.498

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[1] The estimations of CO_2 in blood and plasma were carried out by Mr. K. L. Gad-Andersen by the method of Haldane as modified by Dr. G. Lilliestrand in the laboratory of Professor A. Krogh. A paper by Dr. Lilliestrand describing the method will be published shortly.

Experiment 2.—Temperature 14°C.; Blood: oxygen capacity 15.9 volumes per cent, 0.436 per cent of NaCl.

CO_2 tension, *mm.*	1.1	12.2	43.1	219.0	709.0
NaCl in plasma, *per cent*	0.591	0.551	0.526	0.509	0.479

Experiment 3.—Temperature 15.5°C.; Blood: oxygen capacity 15.2 volumes per cent, 0.460 per cent of NaCl.

CO_2 tension, *mm.*	0.07	4.8	20.4	52.6	78.4
NaCl in plasma, *per cent*	0.600	0.577	0.552	0.543	0.535

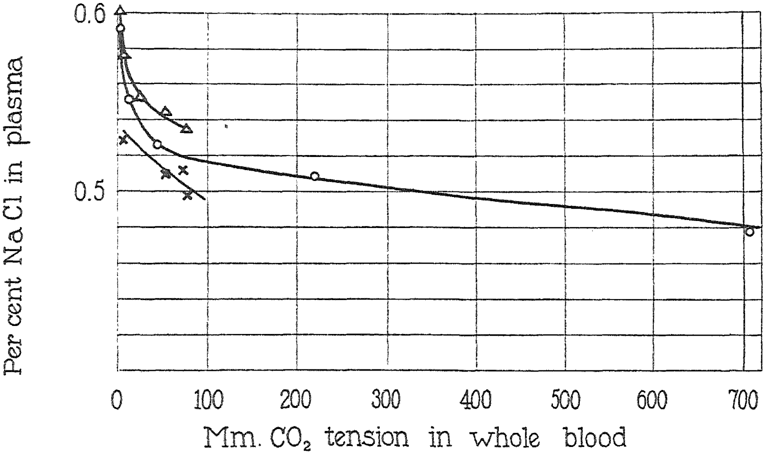

FIG. 1. Curves showing effects of varying amounts of CO_2 on the chlorides of the plasma. X = Experiment 1; O = Experiment 2; Δ = Experiment 3.

In Fig. 1 the results are drawn graphically (carbon dioxide tension as abscissæ and percentage of NaCl in plasma as ordinates). The steep rise of the curve at the low CO_2 tensions refutes Overton's criticism of Hamburger's experiment. When a change in the CO_2 tension from 0.07 to 4.8 mm. makes a measurable quantity of chloride pass from the plasma into the corpuscles, it is impossible that the chloride transfer is due to an injury to the corpuscles by the carbon dioxide. At the high CO_2 tensions the curve is less steep but not parallel to the abscissa axis. The shape of the curve has much conformity with the inverse shape of the curve representing the CO_2-combining power of the blood at various CO_2 tensions. Even at the high CO_2 tensions the plasma contains more chloride than the blood; that is, the chloride content of the corpuscles is never so great as that of the plasma. The distribution of chloride in the blood is never uniform.

The results make it evident that estimation of chlorides in the plasma or serum from blood which has been kept in open receivers must give too high results, because chlorides have passed into the plasma (or serum) on account of the decreasing CO_2 tension.

Experiments 4 and 5 deal with the question of whether the cause of the wandering of the blood chlorides lies in the plasma or in the corpuscles. The problem was attacked by comparing the influence of changes in CO_2 tension upon a sample of blood and upon a sample of the same blood diluted with its own plasma. The plasma for dilution was produced by centrifuging the blood at about 40 mm. CO_2 tension.

Experiment 4.—Temperature 18.5°C.; Sample A: 44 volumes per cent blood corpuscles, oxygen capacity 19.9 volumes per cent, 0.474 per cent of NaCl.

By centrifuging at 40 mm. CO_2 tension plasma is prepared from Sample A.

Sample B is prepared by adding 1 volume of Sample A to 1 volume of its plasma. Sample B: 22 volumes per cent blood corpuscles, oxygen capacity 9.9 volumes per cent, 0.522 per cent of NaCl.

CO_2 tension, *mm*	41.9	47.3	602	681
NaCl in plasma from Sample A after saturating, *per cent*	0.564		0.509	
NaCl in plasma from Sample B after saturating,* *per cent*		0.565		0.545
NaCl in blood corpuscles from Sample A after saturating,* *per cent*	0.36		0.43	
NaCl in blood corpuscles from Sample B after saturating,* *per cent*		0.36		0.43

* The percentage of NaCl in the red blood corpuscles is calculated from the percentage of NaCl of the blood and the plasma by means of the known volume of the red corpuscles (estimated by the hematocrit).

Experiment 5.—Temperature 14°C.; Sample A: 33 volumes per cent blood corpuscles, oxygen capacity 15.0 volumes per cent, 0.442 per cent of NaCl.

Sample B prepared by adding 1 volume of Sample A to 2 volumes of its own plasma. Sample B: 11 volumes per cent blood corpuscles, oxygen capacity 5.0 volumes per cent, 0.483 per cent of NaCl.

CO$_2$ tension, *mm*.	1.2	1.6	34.3	36.7	505	694
NaCl in plasma from Sample A, *per cent*	0.536		0.511		0.460	
NaCl in plasma from Sample B, *per cent*		0.519		0.500		0.491
NaCl in corpuscles from Sample A, *per cent*	0.25		0.31		0.41	
NaCl in corpuscles from Sample B, *per cent*		0.27		0.32		0.42

In Experiment 4 the percentage of NaCl at about 40 mm. CO$_2$ tension was the same in plasma from the original blood (Sample A) and in plasma from the diluted blood (Sample B). In the corpuscles the percentage of NaCl also was the same in Samples A and B at about 40 mm. CO$_2$ tension. When the two samples of blood were saturated with a much higher CO$_2$ tension (about 600 to 680 mm.) the percentage of NaCl fell in the plasma of both, but much more in the plasma from the original blood, Sample A (from 0.564 to 0.509 per cent), than in the plasma of the diluted blood, Sample B (from 0.565 to 0.545 per cent). In the red corpuscles, on the contrary, the percentage of NaCl rose with an equal amount in corpuscles from original and from diluted blood (in either from 0.36 to 0.43 per cent).

Experiment 5 shows the same, and furthermore that by lowering the CO$_2$ tension (from 35 to 1 mm.) the percentage of NaCl rose more in plasma from the original (from 0.511 to 0.536 per cent) than in plasma from the diluted blood (from 0.500 to 0.519 per cent), but coincident with this the percentage of NaCl of the corpuscles fell in each sample by an equal amount.

These results make it evident that the shifting of the blood chlorides is caused by some properties of the red blood corpuscles. A given change in the CO$_2$ tension always produces a given change in the percentage of NaCl of the blood corpuscles, and the percentage of NaCl in the plasma is secondarily regulated by this.

An objection could be raised against the calculation of the percentage of NaCl of the corpuscles, Hamburger (1902) having proved that a change in CO_2 tension affects the volume of the corpuscles. But even a considerable error in the estimation of the volume of the blood corpuscles would not interfere with the results to any noticeable degree.

The cause of the exchange of chloride between blood plasma and blood corpuscles must be looked for in the red corpuscles. In the next section we will consider which constituent of the red corpuscles is involved in this reaction.

Coincident Exchange of Carbon Dioxide and Chloride Ions.

In a recent paper Hasselbalch proved that the hydrogen ion concentration of the blood at a given CO_2 tension is identical with that of a solution of sodium bicarbonate containing the same amount of combined carbon dioxide as the blood and under the same CO_2 tension. But with a change in the CO_2 tension the blood behaves differently from a solution of sodium bicarbonate. The sodium bicarbonate solution contains the same amount of combined carbon dioxide at all CO_2 tensions, but at extremely low tensions some monocarbonate is formed. In blood, however, the amount of combined carbon dioxide increases with increasing CO_2 tension as if the amount of bicarbonate in the blood were increasing too. Hasselbalch explains this by the physicochemical qualities of hemoglobin acting as an amphoteric electrolyte, which has acid qualities so that at low CO_2 tensions it can displace carbon dioxide from the bicarbonate. At high CO_2 tensions hemoglobin assumes alkaline qualities and combines with carbon dioxide in amounts that increase with the CO_2 tension.

Hasselbalch's experiments were performed with solutions of hemoglobin and sodium bicarbonate. The question is how the hemoglobin in the blood can influence the bicarbonate of the plasma as in a solution of hemoglobin, while in the blood the hemoglobin is confined to the corpuscles. This question is explained by the fact that the chloride ions and other anions represent a link between the constituents of the corpuscles and those of the plasma.

When an increase of the CO_2 tension makes chloride pass from the plasma to the corpuscles, the plasma coincidently with this turns more alkaline, and consequently can combine with more carbon dioxide. Gürber has explained this fact in the following way: The carbon dioxide sets free a certain amount of HCl from the chlorides of the plasma; this HCl passes into the corpuscles and leaves the basic components of the chlorides in the plasma to combine with more carbon dioxide. The explanation of Gürber translated into the language of modern chemistry might be worded as follows: Carbon dioxide displaces anions, especially chloride ions, from plasma into corpuscles. Coincidentally with this an equivalent amount of other anions, *viz.* hydroxyl ions, passes from the corpuscles into the plasma. This exchange turns a part of the NaCl of the plasma into NaOH, which combines with some of the free carbon dioxide to form bicarbonate. Koeppe and Hamburger (1902) express the same thing in another way which I do not prefer.

Of course other explanations are possible, especially since Hamburger and Bubanovic have shown that it is likely that the blood corpuscles are permeable not only to anions but also to cations. The matter can be settled by experiments dealing with the minute quantitative relation between chloride and bicarbonate in plasma and corpuscles.

Experiment 6.—Blood was saturated with air at a very low CO_2 tension (0.08 mm.) and centrifuged. Consequently the plasma was very rich in chloride (0.580 per cent of NaCl). Then a sample of the plasma and a sample of the original blood were saturated at a high CO_2 tension (162 mm.). Of course this did not affect the percentage of NaCl of the plasma, but in the blood the plasma became poorer in chloride (0.476 per cent of NaCl), 0.104 per cent of NaCl having passed into the corpuscles.

	NaCl	CO_2
	per cent	*vol. per cent*
Blood at 0.08 mm. CO_2 tension, in the plasma........	0.580	
Plasma " 163 " CO_2 "	0.580	55.6
Blood " 162 " CO_2 " in the plasma...	0.476	103.0
Difference..	−0.104	+47.4

At 163 mm. CO_2 tension the plasma contained 55.6 volumes per cent of CO_2. But the blood saturated at the same CO_2 tension (162 mm.) contained in its plasma (not in the entire blood) 103.0 volumes per cent of CO_2; *i.e.*, 47.4 volumes per cent of CO_2 more than the original plasma separated from the corpuscles at the low CO_2 tension contained at the same CO_2 tension. In going from the low to the high CO_2 tension, 0.104 per cent of NaCl disappeared from the plasma. But coincidently with this the plasma was able to bind 47.4 volumes per cent of CO_2 more. 0.104 per cent of NaCl is equivalent to 39.4 volumes per cent of CO_2; that is, the NaOH formed by the disappearance of chloride ions from the plasma almost completely accounts for the increased amount of carbon dioxide with which the plasma was able to combine.

In Experiment 6 the chloride ions displaced by carbon dioxide from the plasma almost completely account for the increased carbon dioxide-combining power gained by the plasma, but not quite completely, 8 volumes per cent of CO_2 failing to be accounted for. This small amount is probably accounted for by anions other than chlorides passing from the plasma into the corpuscles (de Boer, Hamburger (1918)).

In some experiments on the same problem, Van Slyke and Cullen[2] found that the Cl change did not so closely approximate the CO_2 change. In their experiments other methods were used, and the CO_2 tension in one determination was computed and not gained by analysis. But neither of these facts can account for the considerable difference in the results, which at present I am unable to explain.

If my result holds good, it explains in what way the hemoglobin, although confined to the corpuscle, can influence the CO_2-combining power of the plasma. When the plasma at higher CO_2 tensions binds more CO_2 it is due to the anions passing into the corpuscles to be bound by the hemoglobin, leaving the cations in the plasma to combine with an amount of carbon dioxide equivalent to the amount of disappeared anions.

In Experiment 7 the distribution of chlorides and carbon dioxide at different CO_2 tensions between plasma and corpuscles has been estimated, the NaCl and the bound CO_2 being estimated in the plasma as well as in the blood. The amounts in the corpuscles were calculated from these estimations and from the known volume of the corpuscles. Furthermore the hydrogen ion concentration has been calculated for blood, plasma, and corpuscles by the method of Hasselbalch from the amounts of free and of bound carbon dioxide.

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[2] Van Slyke and Cullen, pp. 343, 344.

Experiment 7.—Temperature 17°C.; 38 volumes per cent blood corpuscles.

CO_2 tension, *mm*		0.08	6.1	39.1
NaCl in	blood, *per cent*	0.456	0.456	0.456
	plasma, *per cent*	0.578	0.533	0.511
	corpuscles, *per cent*	0.260	0.334	0.369
CO_2 bound in	blood, *vol. per cent*	20.8	37.9	65.5
	plasma, *vol. per cent*	23.4	42.6	67.6
	corpuscles, *vol. per cent*	16.6	27.6	62.0
pH in	blood	9.61	7.92	7.31
	plasma	9.64	7.94	7.35
	corpuscles	9.57	7.85	7.30

Experiment 7 shows, as the preceding ones, that with increasing CO_2 tension the amount of NaCl in the plasma decreases and the amount of CO_2 increases. But Experiment 7 shows a new fact too; namely, that not only does the percentage of NaCl in the blood corpuscles increase with increasing CO_2 tension, as is already known, but the amount of carbon dioxide in the corpuscles also increases with increasing CO_2 tension. Although anions at the higher CO_2 tensions pass into the corpuscles the latter do not, as might be expected, become more acid, but on the contrary more alkaline, being able to combine with more carbon dioxide. In the plasma more bicarbonate is generated by the disappearance of anions. In spite of the fact that the corpuscles received the anions, the amount of bicarbonate in the corpuscles apparently increased too. As a result of these changes the hydrogen ion concentration at the CO_2 tensions of the experiments is almost constant in the plasma and the corpuscles, the corpuscles being a little more acid. At very high CO_2 tensions I have not succeeded in getting reliable results.

The explanation of Gürber evidently does not suffice to settle the problem. But when this explanation is combined with Hasselbalch's interpretation of the hemoglobin as an amphoteric electrolyte, the solution of the problem is simple. The more the CO_2 tension (and the hydrogen ion concentration) increases, the more the hemoglobin turns alkaline and the more its power to combine with CO_2 increases. But carbon dioxide ions are anions, as are the chloride ions, and the same property of the hemoglobin which explains its behavior toward carbon dioxide explains also the exchange of chloride ions. Chloride ions and carbon dioxide passing into the corpuscles at increasing hydrogen ion concentration, *viz.* CO_2 tension, are parallel phenomena and due to the same property of the hemoglobin. The difference is due only to the behavior of the plasma, which in a saturater is able to combine with new carbon dioxide from the air of the saturater, replacing the amount which has passed into corpuscles; the plasma is not able to replace the amount of chloride ions which have disappeared, because chloride ions do not exist in the air. This is the explanation, too, why the shape of the curve representing the exchange of chloride ions at different CO_2 tensions as mentioned has conformity with the inverse shape of the curve representing the CO_2-combining power of the blood at various CO_2 tensions.

In a recently published paper[3] Hasselbalch and Warburg fully agree with this interpretation of the facts.

I have made some experiments to see if the chlorides had any influence upon the total amount of carbon dioxide with which the hemoglobin can combine. Experiment 8 is typical.

Experiment 8.—Red blood corpuscles were deprived of their content of chlorides by washing three times with a solution holding 7.3 per cent of cane sugar and 0.185 per cent of sodium bicarbonate. Previous experiments had shown it impossible to deprive corpuscles of all chlorides by washing with isotonic cane sugar solution without addition of a slight amount of bicarbonate to make the solution faintly alkaline. Then the bicarbonate adhering externally to the corpuscles was removed by washing three times with 8.5 per cent cane sugar solution. An analysis was then made and showed no trace of chlorine in the corpuscles. To 32 cc. of the suspension of corpuscles were added 10 cc. of 1.3 per cent sodium bicarbonate solution. This was called Mixture A.

To 20 cc. of Mixture A were added 10 cc. of 8.5 per cent cane sugar solution. This was called Mixture B.

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[3] Read at the same meeting of the Biological Society of Copenhagen as the present paper.

To 20 cc. of Mixture A were added 10 cc. of 0.9 per cent NaCl solution. This was called Mixture C.

Mixtures B and C contained 53 volumes per cent of carbon dioxide combined as bicarbonate.

Samples of Mixtures A and B were then saturated at 17°C. with air containing 29 per cent of carbon dioxide, and other samples with atmospheric air containing 0.03 per cent of carbon dioxide. The amount of carbon dioxide in the various samples was estimated.

CO_2 tension, *mm*.	0.2	218.0
CO_2 in Mixture B, *vol. per cent*	53.9	135.5
CO_2 " " C " " "	51.2	142.6

The results show that chloride-free red blood corpuscles combine with about the same total amount of CO_2 at various CO_2 tensions whether suspended in cane sugar solution or in NaCl solution.

Chlorides in the plasma interfere only with the distribution of carbon dioxide between plasma and corpuscles and not appreciably with the total amount of carbon dioxide, with which chloride-free corpuscles (and presumably the blood) are able to combine.

SUMMARY.

1. The influence of various CO_2 tensions upon the distribution of chlorides between blood plasma and red blood corpuscles was experimentally investigated and the results are represented in curves showing conformity with the curve representing the CO_2-combining power of the blood at various CO_2 tensions.

2. I have been able to confirm, with a different method, the results of Van Slyke and Cullen's experiments on the influence of the CO_2 tension on the partition of the chlorides between plasma and red blood corpuscles.

3. Experimental evidence is presented to show that the cause of the exchange of chloride ions and other anions between plasma and corpuscles is in the red corpuscles and not in the plasma.

4. The amount of chloride ions displaced from the plasma into the corpuscles by increasing CO_2 tension from 0.08 to 162 mm. almost completely accounts for the increased CO_2-combining power coincidently gained by the plasma. My experiments on this point (Experiment 6) do not agree quantitatively with those of Van Slyke and Cullen.

5. Increasing CO_2 tension increases both the CO_2-combining power of the plasma and that of the red corpuscles. The hydrogen ion concentration remains almost the same in plasma and corpuscles at different CO_2 tensions; it is only slightly higher in the corpuscles than in the plasma.

6. If the explanation of Hasselbalch of the properties of the hemoglobin is accepted, it is pointed out that this interpretation explains not only the increasing CO_2-combining power of hemoglobin at increasing CO_2 tension, but also the passing into the red corpuscles of chloride ions (and other anions) at increasing CO_2 tension, the two phenomena being parallel and due to the same property of the hemoglobin. This interpretation accounts for all observed facts.

7. The chlorides of the blood influence only the distribution of carbon dioxide between plasma and red corpuscles, and not appreciably the total amount of carbon dioxide with which the blood is able to combine at various CO_2 tensions.

I wish to thank Professor A. Krogh for much advice and assistance in the experiments.

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A QUANTITATIVE METHOD FOR DETERMINATION OF VITAMINE.

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INTRODUCTION.

In a previous paper[1] it was shown that yeast needs for its nutrition a substance of unknown nature, and evidence was presented that this substance is the beri-beri-preventing vitamine. Shortly after, Bachmann[2] published a paper in which the same conclusion was drawn using a different method of study. It was stated that not only does the growth of single yeast cells indicate presence of vitamine but the rate of growth is dependent on the quantity of vitamine present, and hence it was hoped that the method would be of quantitative value. As a matter of fact, the number of cells produced from one in 18 hours under given conditions is directly proportional to the amount of vitamine added. This test, while of value quantitatively, having been used successfully by a number of people, is not ideal in that it requires some practice to apply it; it introduces a personal factor to quite an extent and is a strain on the eyes if applied regularly. A method is now presented which is based upon a gravimetric determination and measures the growth of a very large number of cells rather than a few which have been isolated, and hence the variations in results are not so large.

The gravimetric method is more accurate than the single cell method; it is more easily applied and the personal factor is largely eliminated. An outline of this method is given first, followed by the justification of the various steps and a general discussion of its application.

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[1] Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.

[2] Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.

Method.

The following solution is used as a stock solution:

20.0 gm. of cane sugar.
3.0 " " $(NH_4)_2SO_4$.
2.0 " " KH_2PO_4.
1.5 gm. of asparagine.
0.25 " " $CaCl_2$.
0.25 " " $MgSO_4$.
All dissolved in 1 liter of distilled water.

100 cc. of this solution are put into a 500 cc. Erlenmeyer flask and to it is added a known amount of the solution to be tested. The volume is then made up to 110 cc., the flask is plugged with cotton, sterilized or pasteurized to kill all vegetative organisms, cooled, and its temperature brought to 30°C. in an incubator.

A yeast suspension is made by weighing out 0.300 gm. of fresh Fleischmann's yeast (small cake in tin-foil) taken from the center of the cake; this is made into a paste with a very small amount of water and suspended finally in 1 liter of sterile water. 1 cc. of this suspension well shaken and freshly made is introduced into the culture medium with a sterile pipette. By this means 0.3 mg. of yeast is used for seeding.

The flasks are then put into the incubator and the yeast is allowed to grow undisturbed 18 hours at 30°C., when the growth is stopped by the addition of a little formaldehyde solution. If the seed yeast has not been kept in a refrigerator continuously since manufacture, a very rapidly growing wild yeast may appear to a slight extent, floating on the surface of the solution. Practically all this should be removed by lifting it from the surface with a small piece of fine copper gauze attached at right angles on the end of a glass rod or tube. This removes practically none of the other yeast, most of which settles to the bottom.

After removing the wild yeast, if any is present, the yeast is filtered off on a weighed Gooch crucible, prepared with paper underneath the asbestos, washed thoroughly with water, finally with a little alcohol, dried for 2 hours at 103°C., and weighed after an hour's cooling. The Gooch crucibles cannot be used repeatedly without treatment as the dried yeast loses weight on washing with water.

If no vitamine is added to the solution the yeast produced will be about 2.5 mg. The amount of growth above that of the blank is, within limits, directly proportional to the amount of vitamine put into the solution. The curve in Fig. 1 indicates the character of the results obtained in the first experiments carried out according to the method outlined.

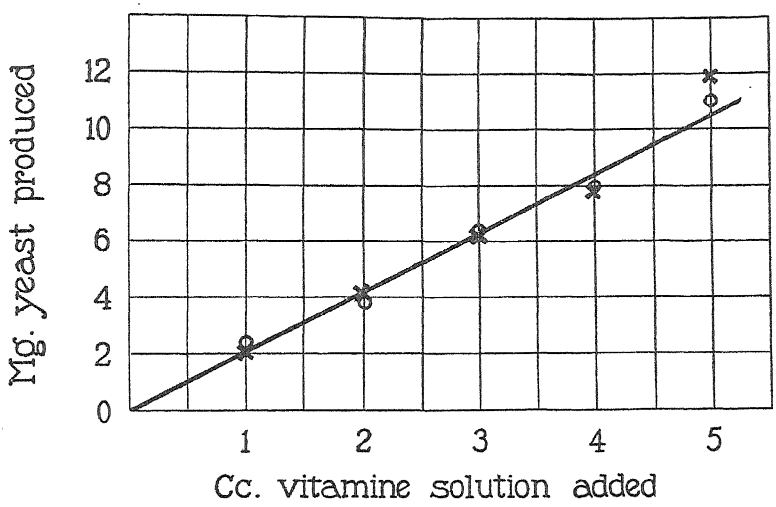

FIG. 1. The points marked with a cross and those marked with a circle were obtained on different days with different samples of yeast. The "Mg. yeast produced" applies to the amount after subtraction of the blank. The "Vitamine solution" contained 1.5 mg. of crude material per cc. For some distance above the upper limits of the curve there is still a proportionality, provided the material tested is free from inhibiting substances.

Expression of Results.

The results may be expressed numerically and the number represents the relative purity of the preparation tested or the value of the material tested as a source of this vitamine. The "vitamine number" of a material may be defined as the number of milligrams of yeast produced by the addition of its extract minus that produced in a control solution, under given conditions and within certain limits, computed to 1 gm. of the original material tested.

Thus if 0.100 gm. of a preparation produces 8.0 mg. of yeast more than a control, its vitamine number computed to 1 gm. is 80. If 0.05 gm. of the preparation is used as a duplicate, approximately 4.0 mg. more yeast are produced than in the control. Computing this to 1 gm., the vitamine number of the preparation is 80, the same as before. It is obvious that this form of expression of results makes possible the comparison of various preparations made at different times and in different localities. By simple water extraction of yeast (such as that used for seeding) and testing the extract obtained we get a vitamine number computed to 1 gm. of dry yeast, which is in the neighborhood of 500. Protein-free milk gives a value of about 12 computed to 1 gm. of the original milk, or 92 computed to the original milk solids. These are merely examples showing the order of magnitude of the numbers.

DISCUSSION.

By watching the growth of single cells under given conditions, which need not be explained in detail, results were obtained which were quantitative in character. The value of a preparation can be expressed in terms of the number of cells produced from one cell under certain conditions, calculated to 1 gm. of substance. The term "vitamine number" as used above is approximately one-twentieth of this value obtained by the single cell method.

By this method of study a number of facts were discovered which have an important bearing on the method under discussion. It has long been supposed that the addition of protein-split products to a medium containing ammonium salt as the sole source of nitrogen improves the medium for yeast. I have found it very difficult to prepare a protein digest free from vitamine which can be made uniformly, and which is a favorable nutrient for yeast. Enzymes cannot be safely used for hydrolysis for this purpose as the enzyme preparations may contain a considerable amount of vitamine. On the other hand, it was found by watching single cells that when ammonium sulfate is supplemented with organic nitrogen, free from vitamine, faster growth may result in the presence of a given amount of vitamine than without the organic nitrogen. It was found that accelerated growth is produced by the addition of a small amount of asparagine to the medium containing ammonium sulfate. The asparagine furnishes amide and amino nitrogen in addition to the ammonia nitrogen already present. A solution which contains ammonium sulfate *and* asparagine is not improved by the addition of a mixture of amino-acids. In an experiment with the single cell method, a mixture of purified amino-acids, alanine, leucine, tyrosine, aspartic acid, glutaminic acid, cystine, histidine, and tryptophane, was added to a medium containing asparagine and a small amount of vitamine to see if the amino-acids improved the medium. The blank with no amino-acids present produced an average of 120 cells from one cell in 18 hours. With 5.0 mg. of the amino-acid mixture the average growth was 108 cells and with 25.0 mg. the average was 96 cells. From these results the conclusion was drawn that nothing except vitamine in the small amounts used will materially improve the medium which already contains asparagine in addition to ammonium sulfate.

It was found by studying the growth of single cells that if an 18 hour incubation period was allowed the growth in this time is directly proportional to the amount of vitamine put into the medium. The 18 hour period was successfully carried over to the gravimetric method here outlined.

When the single cell method is used, commercial yeast can be used to very great advantage,[8] being remarkably uniform in strength and easily suspended for the isolation of single cells. For the gravimetric method also commercial pressed yeast with uniform vitality, moisture content, etc. is very valuable as it makes possible a uniform seeding of the media both in quantity and quality of the yeast. Obviously no bakers' yeast is absolutely free from foreign organisms, but the foreign organisms are very few and offer no serious handicap, as the results indicate. The wild yeast referred to which occasionally appears on the surface of the solution would not have to be considered if it was not for the fact that it multiplies with enormous rapidity even in a weak medium. It does not appear even when quite old yeast is used, if the yeast has been kept at low temperature.

The method as here outlined applies to solutions; that is, the vitamine to be tested must be in solution. No method of extraction is outlined. The fact that nothing except vitamine (especially in small amounts) is able to produce more rapid growth shows that the vitamine does not need to be prepared especially free from other materials, as impurities in all probability will have no accelerating effect. The total amount of material added ordinarily will be much less than 0.1 gm. In using this method for vitamine determinations precautions must be taken that the vitamine is extracted quantitatively from the material in question. Investigators have found that this is difficult to do, especially from some materials. In the handling of the extracts care must be taken that toxic substances are not introduced or formed which will counteract the effect of the vitamine which may be present. This is true also of animal feeding experiments where poisonous substances must be avoided. Treatment of material with acid especially at high temperature or overheating in the dry condition frequently produces something toxic to yeast, while treatment with alkali may destroy the vitamine. The vitamine in some extracts is destroyed to an appreciable extent if kept at the boiling temperature an hour or more. An autolysate of yeast is not a desirable material as a yeast nutrient as inhibiting substances appear to be present. If the amount of growth produced is not proportional to the material added, but falls off with the larger quantity, it is good evidence that something toxic is present, which becomes more potent in the higher concentration and counteracts the effect of the increase in vitamine. This can be confirmed by microscopic examination of the yeast produced, which reveals small granular irregular cells or other abnormalities. It is mainly due to the possible presence of toxic substances that two dilutions should be used for a determination. If toxic substances are present the results from the lower concentrations are more reliable.

It is believed that this method will be of value in the solution of various problems connected with this vitamine. The ultimate source of this water-soluble vitamine, whether it is the green plant or the bacteria of the soil or other organisms, is not settled. Recently, however, evidence has been obtained that when yeast is provided with a certain amount of vitamine it is unquestionably able to build up more. In other words it has some synthetic powers which have also been attributed to animals. The function of this vitamine in animal and plant life, where it seems to occur almost universally, is very obscure. It is a suggestive fact, however, that in some of its properties, solubility, heat stability, alkali destruction, dialyzability, etc., this vitamine bears a resemblance to the coenzyme of alcoholic fermentation.[3]

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[3] For general discussion see Harden, A., Alcoholic fermentation, Monographs on biochemistry, London, New York, Bombay, and Calcutta, 1911, 59-69.

SUMMARY.

A quantitative method for determination of the antineuritic or water-soluble vitamine is presented, which is based upon a gravimetric determination.

A numerical expression of results is suggested whereby the vitamine content of any material can be rated in terms of milligrams of yeast produced under given conditions.

I wish to express thanks to Professor F. C. Koch and Dr. R. E. Lee for help given in carrying out this work.

MODIFICATION OF THE VAN SLYKE METHOD FOR DETERMINING ARGININE.*

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(Received for publication, April 14, 1920.)

Confirming the observation of Osborne, Leavenworth, and Brautlecht[1] that arginine liberates one-half of its nitrogen as ammonia when boiled with concentrated alkali, Van Slyke[2] has made use of a Folin bulb set on top of a tall Liebig condenser to absorb and confine to the system such ammonia as is liberated during the boiling. This method has been used extensively in this laboratory, but from time to time there has been occasion to note the difficulties entailed in its application. The most obvious disadvantage is the possibility of bumping which is likely to occur when such a concentrated solution is boiled for any great length of time. When it does occur, the standard acid in the bulb may be ejected from the bulb or be drawn back into the condenser.

To eliminate these objections and especially to facilitate the securing of accurate results, the apparatus shown in Fig. 1 has been devised and applied with much success. By its use, bumping is entirely eliminated and the ammonia as formed is drawn over into the standard acid by a *slow* current of air. The excess of acid can thus be titrated at once at the termination of the digestion. By the use of N/14 acid, values in cc. synonymous with mg. of nitrogen are obtained directly. In the aspiration it is necessary to use care not to mistake passage of the air through the alkali for ebullition and not to aspirate at too rapid a rate. In other respects the technique used by Van Slyke has been closely followed.

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*Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908–09, xxiii, 194.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15; 1915, xxiii, 411.

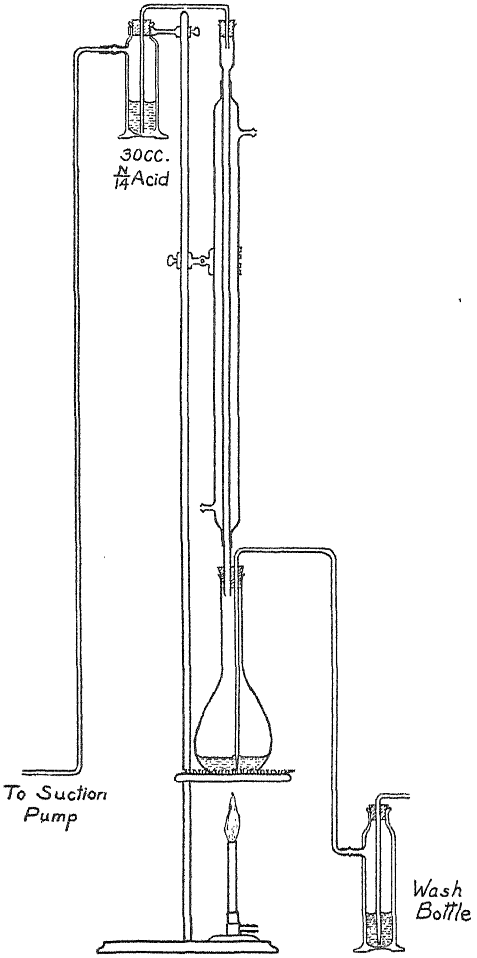

FIG. 1.

FURTHER DATA CONCERNING THE ALLEGED RELATION OF CATALASE TO ANIMAL OXIDATIONS.

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(Received for publication, April 15, 1920.)

Not long since one of us (1) presented evidence which indicated that the causal relation between the catalase concentration of the blood and the intensity of animal metabolism, alleged by Burge (2) to exist, was far from being conclusively demonstrated by his data. A simpler explanation supported by experimental results was presented.

Burge's conclusions were drawn from a certain parallelism existing between the factors noted. As far as the authors are aware he has made no measurements of the intensity of metabolic activity in the course of any of his experiments. It may be true that metabolic intensity and catalase content do vary in the same direction under the conditions he describes but in many and possibly all of his experiments in which these parallel variations in catalase content and metabolic intensity are in harmony with his idea an additional variable factor is introduced; *viz.*, the number of red cells. Inasmuch as the catalase of the blood is contained in the cells, particularly in the erythrocytes, any significance that Burge's experiments really possess is that under the conditions imposed by him the red cell content of the blood varies and with it the catalase as a necessary consequence. The variations in catalase content are, therefore, secondary in nature and bear no necessary relation to the rate of metabolism. This is very different from the unwarranted conclusion that catalase is the oxidizing enzyme of the body.

It is possible to vary the intensity of animal metabolism to a great extent without observing any noteworthy change in the red cell content of the blood. This being true it becomes possible to test Burge's conclusions directly by determining whether, as a matter of fact, there is any change in the catalase content of the blood when the red cell count is constant and the rate of metabolism varied. The result of such a test is the reason for this communication.

Measurements were made of the carbon dioxide production and hemoglobin and catalase contents of rabbit and cat blood drawn to correspond as nearly as possible to successive periods of normal and high metabolism. The apparatus described by Haldane (3) was employed for the carbon dioxide determinations. The high metabolism of the one period was induced in the case of the rabbits by surrounding the animal chamber with ice and partially wetting the fur of the animals and in the case of the cat experiment by the use of a freezing mixture only. Before the beginning of the collection of carbon dioxide, air was drawn through the apparatus with the animal in place for from 11 to 15 minutes. The current varied between 4 and 6 liters per minute in the different experiments but the rate was practically constant in any one of them. Thus the accumulation of carbon dioxide in the chamber was prevented since in the fore periods and in the intermediate period of the last experiment there was ample opportunity for an equilibrium to be established between the production and removal of the gas. Therefore the carbon dioxide collected in the subsequent periods closely approximates the actual production in those periods. In the rabbit experiments the period of high metabolism followed that of normal metabolism while in the cat experiment the order was reversed. The animals varied in size, which accounts partly for the differences in the rates of metabolism noted. They filled the chamber almost completely so that there was very little voluntary activity. Catalase estimations were made by the method already described (1); hemoglobin determinations were made by Palmer's (4) procedure. The results of the experiments are shown in Table I.

These six experiments appear to demonstrate conclusively that there may be great variations in the rate of animal metabolism without any corresponding change in the catalase content of the blood. According to Burge's hypothesis variations in catalase content and carbon dioxide production should be parallel. The statement is warranted, therefore, that the parallelism between metabolic intensity and the catalase content of blood which sometimes exists, but which is not found here, does not prove any intimate relation between the two. On the other hand, it appears that the data here presented suggest the absence of such an interrelation in so far as it is reasonable to expect variations in the oxidizing capacity of the tissues to be reflected in the concentration of oxidizing enzymes in the blood.[1]

TABLE I.

Animal.	Period.	Catalase.	Hemoglobin.	CO_2	Remarks.
		cc.	*per cent*	*gm.*	
Rabbit 1.	Room temperature.	131,130	84	1.34	45 min. periods. Blood drawn from ear immediately preceding Period A and at end of Period B.
	Low “	133,130	90	3.56	
“ 2.	Room “	115,116	83	0.69	30 min. periods. Blood as above.
	Low “	125,129	83	2.16	
“ 3.	Room “	124,122	71	1.18	30 min. periods. Blood as above. Temperature of chamber lowered further in Period B.
	Low “ (Period A)	127,117	71	1.69	
	Low temperature. (Period B)	120,121	74	2.20	
“ 4.	Room temperature.	142,136	74	1.70	45 min. periods. Blood as above.
	Low “	128,150	71	2.17	
“ 5.	Room “	173,167	96	1.10	30 min. periods. Blood as above.
	Low “	166,170	95	2.20	
Cat 1.	Low “	212,213	76	4.06	45 min. periods. Blood drawn from femoral vein at end of each period.
	Room “	235,242	81	1.95	

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[1] Since this paper was written Seymour has reported that no direct relation exists between the catalase content of turtle heart muscle and the rate of its beat (Seymour, R. J., *Am. J. Physiol.*, 1920, li, 525).

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THE FERMENTATION OF GLUCOSE, GALACTOSE, AND MANNOSE BY LACTOBACILLUS PENTOACETICUS, N. SP.*

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The pentose-fermenting bacteria to which we have given the name *Lactobacillus pentoaceticus, n. sp.*[1] exhibit a wide range of activity, both with respect to carbohydrates fermented and products formed. The pentoses, xylose and arabinose, are rapidly and completely broken down to acetic acid, lactic acid, and only the merest trace of other products such as carbon dioxide and alcohol. The fermentation of fructose is not so simple and results in the production of large quantities of carbon dioxide and mannitol in addition to the products formed from xylose.[2] The fermentation of fructose to mannitol is completed in 4 or 5 days, and a secondary fermentation of the mannitol then ensues, the chief products of which appear to be much the same as those derived from fructose. However, the fermentation of mannitol is much slower than that of fructose.

The fermentation of the aldo-hexoses, glucose, galactose, and mannose, differs from the fermentation of fructose in that no mannitol and very little acetic acid are formed, but a new compound, ethyl alcohol, is produced in large quantities. In old cultures a part of the lactic acid formed may be broken down to

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[1] Fred, E. B., Peterson, W. H., and Davenport, A., *J. Biol. Chem.*, 1919, xxxix, 347.

[2] Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, 1920, xli, 431.

acetic acid and carbon dioxide as decomposition products. On the basis of the quantitative data obtained, it is proposed that the main line of the fermentation for the aldo-hexoses may be represented by the equation

$$C_6H_{12}O_6 = CH_3CHOH.COOH + C_2H_5OH + CO_2$$

The small amount of acetic acid found probably results from a secondary fermentation of the lactic acid. The application of quantitative data to this equation will be considered in the experimental part of this paper.

The products formed from the aldo-hexoses by the pentose fermenters, *Lactobacillus pentoaceticus*, indicate a relationship to other organisms such as the "mannite-bacteria" of Gayon and Dubourg, Laborde, Müller-Thurgau and Osterwalder, and *Bacillus coli communis* of Harden, and the lactic acid bacteria of Kayser.[3]

Complete data for all the products formed by different bacteria are meager. Most of the investigational work has been concerned with a measurement of one or two products rather than a balance of all the products. It is, therefore, often difficult to determine the underlying causes that modify the formation of a given product. Frequently the variation in amount of one product is intimately associated with variation in the amount of another product. This interrelation between the products is exhibited very strikingly in the recent work[4] with yeast where glycerol production is paired with the production of acetaldehyde or acetic acid. Likewise, compounds which are termed final products may be the result of secondary fermentations, so that it is impossible to relate them directly to the carbohydrate fermented. A careful balance between the total products formed, the sugar fermented, and the ratios of these products to one another will frequently disclose relations that were unsuspected and help to give a more correct picture of the mechanism of the fermentation.

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[3] Gayon, U., and Dubourg, E., *Ann. Inst. Pasteur*, 1894, viii, 108; 1901, xv, 527. Laborde, J., *Compt. rend. Acad.*, 1904, cxxxviii, 228. Müller-Thurgau, H., *Landw. Jahrb. Schweiz*, 1907, xxi, 230. Müller-Thurgau, H., and Osterwalder, A., *Centr. Bakteriol.*, *2te Abt.*, 1912, xxxvi, 129; 1917–18, xlviii, 1. Harden, A., *J. Chem. Soc.*, 1901, lxxix, 610. Kayser, E., *Ann. Inst. Pasteur*, 1894, viii, 737.

[4] Neuberg, C., and Reinfurth, E., *Biochem. Z.*, 1918, lxxxix, 365; xcii, 234. Neuberg, C., and Hirsch, J., *Biochem. Z.*, 1919, xcvi, 175.

The most comprehensive chemical data on the products formed by organisms similar to *Lactobacillus pentoaceticus* are those of Gayon and Dubourg. They give complete data for the products formed from fructose, glucose, galactose, sucrose, maltose, lactose, and raffinose, and nearly complete data for mannose, sorbose, and xylose.

From glucose, galactose, and mannose they obtained acetic acid, lactic acid, alcohol, carbon dioxide, glycerol, and succinic acid. The proportion of these products varied somewhat with the age of the culture, the kind and amount of nutrients used, and the reaction of the medium. With the exception of glycerol the percentage of sugar represented by the different products falls in the neighborhood of that formed by the pentose fermenters. Their organism fermented raffinose but failed to attack arabinose, mannitol, glycerol, or malic acid, which is just the reverse of what takes place with the pentose fermenters of the group *Lactobacillus pentoaceticus.*

Müller-Thurgau and Osterwalder describe several mannite-forming bacteria which produced acetic acid, lactic acid, and alcohol from glucose and galactose. The relation of these products to one another varied with the organism concerned. Their papers give no data for the amount of gas formed or for the percentage of sugar represented by the end-products. A careful comparison of the pentose fermenters with their organisms shows many points of similarity, and also many differences in respect to fermentation power and ratio of products formed. The most marked differences are the fermentability of mannitol and lactates and the non-fermentability of raffinose by *Lactobacillus pentoaceticus.* Other differences in behavior manifest themselves toward arabinose, xylose, glycerol, and malates. Two of their organisms show high acetic acid production while a third, *Bacterium intermedium,* is more like *Lactobacillus pentoaceticus* in this respect. However, *Bacterium intermedium* ferments raffinose and fails to ferment arabinose, glycerol, and lactic acid.

The fermentation of glucose by *Bacillus coli communis* results in many of the same products and in quantities comparable to those formed by the pentose fermenters. From the data given by Harden, acetic acid, lactic acid, alcohol, and carbon dioxide comprise the major products with small amounts or traces of succinic acid and formic acid. On the basis of the quantitative data obtained, Harden proposes the equation

$$2\ C_6H_{12}O_6+H_2O=2\ C_3H_6O_3+C_2H_4O_2+C_2H_6O+2\ CO_2+2\ H_2$$

The most conspicuous difference between the products formed from glucose by Harden's *Bacillus coli communis* and by *Lactobacillus pentoaceticus* is the very small amount of acetic acid produced from glucose by the latter. As yet no evidence has been obtained with respect to the production of hydrogen by the pentose fermenters.

Kayser[5] made an extensive investigation of the conditions surrounding the production of lactic acid by bacteria, isolated from various materials. One of these, obtained from sauerkraut, possessed the power to ferment a large number of carbohydrates including many of the less common sugars such as xylose, arabinose, melezitose, trehalose, and raffinose. His paper gives no data for the quantity of products formed other than volatile acid, mainly acetic, and lactic acid.

In a later paper Kayser[6] gives quantitative data for the production of acetic acid, lactic acid, ethyl alcohol, carbon dioxide, and mannitol for a number of sugars. These bacteria differ from the pentose fermenters with respect to the kind of volatile acids formed and the ratios of the various end-products.

EXPERIMENTAL.

Fermentation.—The culture solution for fermentation was prepared by dissolving the glucose in a yeast water extract and sterilizing for 30 minutes at 15 pounds pressure. After sterilization, the solution was inoculated, and in certain cases sterilized calcium carbonate added. When calcium carbonate was not used, the acidity which developed during fermentation as shown by the presence of bromocresol purple was neutralized from time to time with sterilized 1 N NaOH.

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[5] Kayser, E., *Ann. Inst. Pasteur*, 1894, viii, 737.

[6] Kayser, E., *Ann. Inst. nat. agron.*, *Series 2*, 1904, iii, 241.

Volatile and Non-Volatile Acids.—At the end of the fermentation period the cultures were made up to a definite volume, centrifugalized to throw down the calcium carbonate, and an aliquot was taken for analysis. The volatile and non-volatile acids were determined by steam distillation and ether extraction, respectively, as previously described. The nature of the volatile acids formed was subsequently determined by subjecting them to a Duclaux distillation and by the barium content of their barium salts. The volatile acid proved to be almost entirely acetic.

The non-volatile acids extracted by the ether were determined by the Moeslinger method[7] for lactic acid and their identity was established by the zinc salt. The non-volatile acid was found to be racemic lactic acid. No evidence of any other non-volatile acid has been secured.

Alcohol.—The alcohol was obtained by saturating the neutralized culture solution with sodium chloride and then distilling over one-half to two-thirds of the aliquot taken. The distillate was oxidized with potassium dichromate and sulfuric acid as described by Dox and Lamb,[8] and then distilled. The acids thus obtained were identified in the same manner as already described for the volatile acids.

Carbon Dioxide.—When carbon dioxide and alcohol were determined, the fermentation was carried on in the apparatus described in a former paper[2] and the absorbed carbon dioxide determined by means of the Van Slyke[9] apparatus for measuring gases.

Fermentation of Glucose in the Presence of Calcium Carbonate.

Fermentation took place readily, but the analytical data showed only small amounts of acetic acid and relatively large amounts of lactic acid. Differences in the ratios between these two acids and data collected from the fermentation of fructose indicated that in old cultures the lactic acid in part was converted into acetic. Large cultures were therefore set up and aliquots were removed and analyzed at different periods in the fermentation.

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[7] Moeslinger, Z. *Untersuch. Nahrungs-u. Genussmittel*, 1901, iv, 1120.

[8] Dox, A. W., and Lamb, A. R., *J. Am. Chem. Soc.*, 1916, xxxviii, 2561.

[9] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Marked increase in the quantity of acetic acid was found while the lactic acid remained stationary or decreased in amount. In an old culture the formation of lactic acid from glucose proceeds at about the same rate as the decomposition of lactic acid into acetic acid. Hence the latter accumulates while the quantity of the former remains stationary or decreases. In the case of Culture 41–11, the acetic has increased from 0.087 to 0.416 gm. per 100 cc., while the lactic remained practically unchanged at 0.717 gm. With Culture 118–8, the increase for acetic is from 0.078 to 0.328 gm., while the lactic shows an actual decrease from 0.713 to 0.637 gm. The data are given in Table I.

TABLE I.

Fermentation of Glucose in the Presence of Calcium Carbonate for Various Lengths of Time. Calculated for 100 Cc. of Culture.

Culture No.	Age of culture.	Weight of glucose.	Volatile acid as acetic.	Non-volatile acid as lactic.	Ratio acetic:lactic.
	days	*gm.*	*gm.*	*gm.*	
41–11	15*	2.00	0.087	0.717	100:824
41–11	30	2.00	0.157	0.722	100:460
41–11	75	2.00	0.416	0.720	100:173
118–8	15*	2.00	0.078	0.713	100:914
118–8	30	2.00	0.112	0.740	100:661
118–8	75	2.00	0.328	0.637	100:194

* Both cultures analyzed at successive intervals during the fermentation.

Attention is called to similar results obtained by Kayser, and Gayon and Dubourg. The former reports almost complete destruction of the lactic acid at the end of 20 days. Gayon and Dubourg found 1.62 gm. of acetic acid and 6.10 gm. of lactic acid in a 9 day culture, and 25 days later found the acetic acid had increased to 3.73 gm., while the lactic acid had decreased to 4.68 gm.

Loss of Alcohol from Fermenting Cultures.

Duplicates of the cultures whose volatile acid content is given in Table I were examined for ethyl alcohol, but the results were either negative or inconclusive. This failure to find alcohol proved to be due to evaporation from the fermentation flask.

The culture solution of 50 cc. volume was contained in a 300 cc. Erlenmeyer flask, stoppered in the usual way with a cotton plug, and after 32 days of fermentation had evaporated to about 40 cc.

Because of the small amount of alcohol found, a second experiment was set up where 500 cc. portions of solution were fermented in liter flasks. At the end of the fermentation period (23 days), the alcohol in the entire culture was distilled out and concentrated to a volume of 200 cc. The alcohol in 50 cc. was determined by the dichromate oxidation method while another portion (100 cc.) was concentrated to 33 cc. by successive distillations. This solution contained sufficient alcohol to give a good burning test and the characteristic iodoform crystals. These results indicate that great loss of volatile products such as alcohol, esters, and possibly acids may occur when flasks are stoppered with loose cotton plugs. In this connection it is of interest to note the loss of acetone from cultures of *Bacillus acetoethylicum* recently reported by Northrop, Ashe, and Senior.[10] The data involved in these fermentations are given in Table II.

TABLE II.

Loss of Alcohol from Flasks Stoppered with Cotton Plugs.

Culture No.	Age of culture.	Weight of sugar.	Volume of fermenting solution.	Volume of flask.	Ethyl alcohol.	Sugar represented by alcohol.
	days	*gm.*	*cc.*	*cc.*	*gm.*	*per cent*
55-9	32	1.00	50	300	0.017	1.7
69-19	32	1.00	50	300	0.010	1.0
41-11	23	20.00	500	1,000	2.301	11.5
118-8	23	20.00	500	1,000	2.608	13.0

Balance of Products from the Fermentation of Glucose.

In these experiments all the products which had been shown to be formed were determined. Culture solutions of 250 to 400 cc. were fermented, and at the end of the fermentation period the various products formed were determined on different aliquots. At the same time the unfermented sugar was determined. The data are given in Table III and bring out some very interesting relations.

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[10] Northrop, J. H., Ashe, L. H., and Senior, J. K., *J. Biol. Chem.*, 1919, xxxix, 5.

1. In general, not more than 70 to 80 per cent of the glucose is fermented. The single exception to this is the 25 day Culture 118–8. The fermentation in the case of the aldo-hexoses is much less complete than in that of the pentoses. The inhibiting factor must be something other than the salts of the acids produced, for the concentration of these is higher with the pentoses than with the aldo-hexoses. No explanation can be given at present for this incomplete fermentation.

2. The sum of the four products determined represent from 85 to 95 per cent of the sugar fermented. The unknown 5 to 15 per cent would include any inaccuracies in the analytical methods, the sugar incorporated in the cells of the organisms, and minor products that have hitherto escaped detection.

TABLE III.

Total Fermentation Products from Glucose. Calculated for 100 Cc. of Culture.

Culture No.	Age of culture.	Weight of glucose ferment-ed.	Acetic acid.	Lactic acid.	Ethyl alcohol.	Carbon dioxide.	Glucose accounted for by products.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
41–11	6	0.80	0.035	0.254	0.156	0.150	74.3
41–11	14	1.22	0.054	0.474	0.248	0.293	87.6
41–11	20	1.05	0.070	0.343	0.197	0.251	82.0
118–8	25	1.92	0.092	0.816	0.440	0.503	96.4

3. The ethyl alcohol and carbon dioxide are produced in nearly equivalent quantities. The slight excess of carbon dioxide over ethyl alcohol may be due in part to a secondary fermentation of lactic acid as will be shown later. The nearly equal quantities of alcohol and carbon dioxide produced by many bacteria have been recorded by different investigators. This observation suggests the possibility that alcohol and carbon dioxide are formed from intermediate products similar in character to those found in yeast fermentation.

From the data given in Table III the relation of the different products to one another has been calculated and expressed as percentage of the total products formed. The figures thus obtained are compared with the percentage relations that should exist if the fermentation proceeded according to the theory already proposed. An examination of the data given in Table IV shows values too low for lactic acid, and too high for carbon dioxide. The presence of 5 to 8 per cent of acetic acid is not allowed for in the theoretical equation. Very good agreement exists between the found and the calculated values for alcohol. The 5 to 8 per cent of acetic acid and excess 3 per cent of carbon dioxide are roughly equal to the deficiency in the value for lactic acid. The decreased ratio of acetic acid to lactic acid in old cultures, and the direct fermentation of lactic acid into acetic acid and carbon dioxide seem to warrant this explanation. The data are given in Table IV.

TABLE IV.

Percentage Distribution of Fermentation Products from Glucose.

Chemical compound.	Culture 41-11.			Culture 118-8.	Average.	Theory.
	6 days.	14 days.	20 days.	25 days.		
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Acetic acid...........	5.9	5.0	8.1	5.0	6.0	0.0
Lactic "	42.7	44.3	40.0	44.1	42.8	50.0
Alcohol..............	26.3	23.2	22.9	23.8	24.0	25.6
Carbon dioxide.......	25.2	27.4	29.1	27.2	27.2	24.4

Fermentation of Galactose.

The products obtained by the fermentation of galactose are the same as those obtained from glucose, but more galactose seems to have been consumed by the bacteria than was the case with glucose. The evidence on this point is somewhat conflicting and does not warrant any positive statement. The organisms seem to use these two sugars with equal ease. The total products formed and the percentage relations of the different products are given in Tables V and VI.

TABLE V.

Total Fermentation Products from Galactose. Calculated for 100 Cc. of Culture.

Culture No.	Age of culture.	Weight of sugar fermented.	Acetic acid.	Lactic acid.	Ethyl alcohol.	Carbon dioxide.	Galactose accounted for by products.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
41–11	33	1.406	0.086	0.522	0.389	0.343	95.3
118–8	10	1.385	0.064	0.475	0.341	0.305	85.5
118–8	33	1.542	0.065	0.545	0.436	0.409	94.4

TABLE VI.

Percentage Distribution of Fermentation Products from Galactose.

Chemical compound.	Culture 41–11.	Culture 118–8.		Average of three cultures.
	per cent	*per cent*	*per cent*	*per cent*
Acetic acid	6.4	5.4	4.5	5.4
Lactic "	39.0	40.1	37.4	38.8
Ethyl alcohol	29.0	28.8	30.0	29.3
Carbon dioxide	25.6	25.7	28.1	26.5

TABLE VII.

Total Fermentation Products from Mannose. Calculated for 100 Cc. of Culture.

Culture No.	Age of culture.	Weight of sugar fermented.	Acetic acid.	Lactic acid.	Ethyl alcohol.	Carbon dioxide.	Mannose accounted for by products.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
41–11	37		0.328	0.324			
41–11	16	0.853	0.097	0.259	0.182	0.191	85.5
118–8	38	0.578	0.084	0.104	0.141	0.173	87.0

TABLE VIII.

Percentage Distribution of Fermentation Products from Mannose.

Chemical compound.	Culture 41–11.	Culture 118–8.
	per cent	*per cent*
Acetic acid	13.3	16.7
Lactic "	35.5	20.8
Ethyl alcohol	25.0	28.0
Carbon dioxide	26.2	34.5

Fermentation of Mannose.

Mannose is generally regarded as a sugar that cannot be easily fermented by lactic acid-producing bacteria, and the results obtained with *Lactobacillus pentoaceticus* agree with this general experience. Only from 30 to 45 per cent of the sugar is consumed after 38 days. The difficulty with which mannose is fermented is probably related to the stereoisomeric configuration of mannose. Its configuration is more unlike the *d*-glucose type of structure than any of the other sugars used; *viz.*, glucose, galactose, xylose, or arabinose. The data for the fermentation products formed are given in Table VII.

The percentage distribution of the various products formed from mannose is given in Table VIII, and reveals a higher production of acetic acid and carbon dioxide, and a lower production of lactic acid than that obtained from either of the other two sugars. The difficult fermentation of mannose evidently forces the bacteria to destroy more lactic acid than is the case with the more readily fermented glucose and galactose.

Fermentation of Lactates.

As has already been pointed out in connection with the fermentation of fructose, mannitol, and glucose, there was an enormous change in the ratio of acetic acid to lactic acid in cultures where fermentation was continued for 50 to 70 days. In some cases there was an actual decrease in the quantity of lactic acid. This could only mean that some of the lactic acid was destroyed in the later stages of the fermentation, and possibly acetic acid was formed as one of the decomposition products. An attempt was therefore made to ferment the lactate directly. In the first experiment 2 gm. of sodium lactate syrup were added to the usual yeast water and inoculated with the pentose fermenters. Growth was apparent in 1 or 2 days; the medium became turbid, the bromocresol purple present indicated a change in reaction, and stained mounts showed the presence of large numbers of bacteria. After 17 days the control and two of the cultures were analyzed for volatile acid and unfermented lactic acid. It was found in the case of Culture 118–8 that the volatile acid had increased from

4.7 cc. of 0.1 N acid in the control to 28.3 cc. in the culture, and the lactic acid had decreased from 113.0 cc. of 0.1 N acid in the control to 91.0 cc. in the culture. The total acidity had remained practically unchanged, being 117.7 cc. of 0.1 N acid for the control and 119.3 cc. in the fermented solution. The volatile acid formed was later identified as acetic acid. After 37 days of fermentation two other cultures of the same series were analyzed and showed a further increase in acetic acid and a corresponding decrease in lactic acid.

TABLE IX.

Fermentation of Sodium Lactate. Calculated for 100 Cc. of Culture.

Culture No.	Age of culture.	Acetic acid.	Lactic acid.	Carbon dioxide.	Ratio acetic: CO_2
Series I.	*days*	*gm.*	*gm.*	*gm*	
Control.	17	0.028	1.016		
41-11	17	0.152	Lost.		
118-8	17	0.170	0.818		
41-11	37	0.226	0.725		
118-8	37	0.242	0.689		
Series II.					
Control.	48	0.026	Lost.		
41-11	48	0.074	1.043	0.034	100:71*
118-8	48	0.077	1.031	0.031	100:61*

* The value of the acetic acid control was deducted before calculating the ratios.

In the second experiment a carbon dioxide absorption bottle was attached to each fermentation flask and the absorbed gas determined at the end of the fermentation period. As judged by the quantity of acetic acid formed, the lactic acid was not decomposed to the same extent as was the case in the first experiment. The ratio of acetic acid to carbon dioxide was calculated and approximates that required for the theory that a molecule of carbon dioxide is formed for each molecule of acetic acid. The complete data calculated in gm. per 100 cc. of culture are given in Table IX.

Identification of Products.

Volatile Acid.—After titrating the volatile acids with 0.1 N $Ba(OH)_2$ the distillate was evaporated to dryness, taken up with about 60 to 70 cc. of hot water, and filtered into 300 cc. Erlenmeyer flasks. The flask was heated to boiling and when cooled was closed with a one-hole rubber stopper containing a carbon filter funnel. The end of the funnel was drawn out to a fine capillary tube. The amount of 1 N H_2SO_4 required to decompose the barium salts was then added through the dropping funnel. No volatile acids could thus be lost from the hot solution. After standing a few hours at room temperature, the barium sulfate was filtered off, the filtrate made up to 110 cc., and subjected to Duclaux distillation. In the case of alcohol, the acid formed by oxidation and subsequent distillation was put through the same manipulation. The distilling constants obtained are given in Table X and agree with that found for acetic acid. In the case

TABLE X.

Distilling Constants of the Volatile Acids Obtained by the Duclaux Method.

Source of volatile acid.	Culture No.	Fractions.									
		10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
Glucose.	41–11*	7.5	15.4	23.7	32.5	41.6	51.1	61.1	72.3	84.8	100
"	41–11†	7.7	15.6	24.1	32.9	42.1	51.7	62.4	73.1	85.5	100
Alcohol from glucose.	41–11	7.1	15.6	24.0	32.8	42.0	51.6	61.9	73.0	85.0	100
Galactose.	118–8	7.9	16.1	24.6	32.3	42.3	51.8	61.9	72.9	85.2	100
Alcohol from galactose.	41–11	7.6	15.6	24.0	32.4	41.8	51.4	61.6	72.7	85.1	100
Mannose.	118–8	8.1	16.4	25.0	33.9	43.1	52.6	62.6	73.5	85.7	100
Alcohol from mannose.	118–8	8.3	16.6	25.0	33.8	42.9	52.5	62.6	73.5	85.7	100
Lactic acid.	41–11	7.8	15.8	24.3	33.1	42.3	51.9	62.2	73.2	85.5	100
Duclaux distilling constant for acetic acid.		7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100

* 20 day culture.

† 75 " "

of mannose, a small quantity of higher acid and alcohol seems to be present. An exact check cannot be expected as the controls always give 3 to 4 cc. of 0.1 N volatile acid. This acid originates either from the yeast water or from the sugars as a result of sterilization. It is also apparent that the alcohol formed in the fermentation is ethyl alcohol.

TABLE XI.

Composition of the Barium Salts of the Volatile Acid.

Culture No.	Compound fermented.	Barium salt of the volatile acid.	Barium sulfate equivalent.	
			Found.	Calculated for acetic acid.
		gm.	*gm.*	*gm.*
41–11	Glucose.	0.4248	0.3830	0.3957
41–11	Alcohol from glucose.	0.4106	0.3722	0.3825
41–11	Galactose.	0.2170	0.1900	0.1973
41–11	Alcohol from galactose.	0.9796	0.8912	0.8951
118–8	Mannose.	0.2056	0.1840	0.1879
118–8	Alcohol from mannose.	0.3406	0.3068	0.3112
41–11	Lactic acid.	0.4418	0.3958	0.4036

TABLE XII.

Water of Crystallization Contained in Zinc Lactate.

Culture No.	Source of salt.	Weight of zinc lactate used.	Water lost.		Water in $Zn(C_3H_5O_3)_2 + 3H_2O$.
		gm.	*gm.*	*per cent*	*per cent*
41–11*	Glucose.	0.4086	0.0696	17.0	18.17
41–11†	“	0.4414	0.0772	17.5	18.17
118–8	Galactose.	0.7126	0.1284	18.0	18.17
118–8	Mannose.	0.1670	0.0288	17.3	18.17

* 20 day culture.
† 75 “ “

As a check on the Duclaux distilling constants, the barium content of the volatile acids has also been determined. The data found are given in Table XI and furnish evidence that the volatile acid is acetic, and the alcohol is ethyl.

The Form of Lactic Acid Produced.—The zinc salt was prepared from the barium salt of the lactic acid formed and the water of crystallization determined. As is well known, the inactive zinc lactate crystallizes with 3 molecules of water, 18.17 per cent. From 17.0 to 18.0 per cent of water was found which indicates that the lactic acid formed is of the racemic type. The appearance of the crystals under the microscope resembled that of *d-l*-lactic acid. The data are given in Table XII.

SUMMARY.

The aldo-hexoses, glucose, galactose, and mannose, are fermented by *Lactobacillus pentoaceticus*, with the production of lactic acid, ethyl alcohol, carbon dioxide, and small quantities of acetic acid.

The acetic acid is probably not a direct fermentation product, but results from a secondary fermentation of the primary product, lactic acid. Evidence for this statement is based on the changing ratio of acetic to lactic with the age of the culture. In the early stages of the fermentation this is about 100:800, while in old cultures, 75 days, this ratio has decreased to 100:200. Not only has the ratio changed, but there has been an actual loss of lactic acid from the fermenting solution.

Further proof of the fermentation of lactic acid to acetic is adduced from the direct fermentation of lactates. The products formed are acetic acid and carbon dioxide.

Glucose and galactose are fermented at approximately the same rate and to the same extent. Mannose is more slowly attacked and less of the sugar is consumed. The unfermented glucose and galactose remaining after 30 to 40 days is about 20 to 30 per cent. In the case of mannose, the amount is from 50 to 70 per cent. The difficult fermentation of mannose results in a corresponding increase in the secondary fermentation of lactic acid to acetic acid.

The difference in the extent of fermentation of glucose, galactose, and mannose must rest on their configuration. The difference in stereoisomerism between an aldose and a ketose as illustrated by glucose and fructose results in the production of ethyl alcohol as one of the major products from glucose, and of acetic acid from fructose. This result is probably due to different conditions of oxidation and reduction operating on the intermediate products of the fermentation.

THE METABOLISM OF SULFUR.

III. THE RELATION BETWEEN THE CYSTINE CONTENT OF PROTEINS AND THEIR EFFICIENCY IN THE MAINTENANCE OF NITROGENOUS EQUILIBRIUM IN DOGS.

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(Received for publication, April 5, 1920.)

The importance of cystine for normal growth of the young white rat has been demonstrated by Osborne and Mendel (1) and more recently by Johns and Finks (2). In consequence of this work cystine is generally recognized as one of the essential amino-acids which must be present in adequate amounts in the diet. In acceptance of this belief most workers in nutrition experiments with hydrolyzed proteins have added cystine to replace that destroyed in the hydrolysis, particularly in acid hydrolysis (3, 4). Concerning the requirement of cystine for maintenance of adult animals, less information is available. Geiling (4) concluded that cystine was necessary for the maintenance of adult white mice.

In a former paper (5), it has been shown that the addition of cystine in small amounts to the food of dogs maintained on a low protein level diminished the loss of nitrogen from the body and favorably influenced the nitrogen balance. Since the addition of tyrosine and glycocoll as typical essential and non-essential amino-acids respectively exerted no influence on the condition of nitrogenous equilibrium under the same experimental conditions, the effect of cystine was considered specific. The work indicated that cystine was the amino-acid which determined the protein minimum for the mixture of proteins fed, *i.e.* beef heart, and furnished strong experimental evidence of the indispensability of preformed cystine in adequate amounts for the maintenance of adult animals. A more definite interpretation of the data was difficult since the cystine content of the mixed protein fed, the various proteins contained in the heart muscle of cattle and in the blood of the coronary vessels, was unknown. Analysis of the mixed proteins isolated from the material fed in the experiments showed a rather low content of sulfur (0.73 per cent).

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* Some of the preliminary work in these experiments was carried on with the aid of Mr. G. E. Simpson.

It was considered desirable to extend the work to include proteins of known sulfur (and cystine) content in order to ascertain to what extent the minimum for these proteins for maintenance of species other than the white rat and mouse was determined by their cystine content. The present investigation was planned to answer as far as possible the following questions. Do pure proteins of known sulfur (and cystine) content vary in their efficiency for the maintenance of nitrogenous equilibrium under conditions of a low protein input according to the relative amounts of sulfur (and cystine) they furnish? Can a protein low in sulfur (and cystine) be made more adequate for nutrition under the same conditions by the addition of cystine, the only definitely proved sulfur-containing complex of the protein molecule?

Casein and serum proteins were chosen for the experimental study as representative cystine-poor and cystine-rich proteins respectively. Casein contains about 0.8 per cent sulfur, but it is probable that only a small part of this sulfur is present as cystine. Attempts to isolate cystine from the hydrolysis products of casein have met with little success. Mörner (6) was able to obtain only 17 mg. of cystine from 100 gm. of casein. Casein on treatment with strong alkali yields about 13 per cent of its sulfur as alkali sulfide (7), which on the assumption that all this sulfide sulfur originates from cystine corresponds to about 0.60 per cent of cystine (1). Serum albumin contains a higher percentage of sulfur than casein and yields about two-thirds of its sulfur as alkali sulfide on boiling with alkali (7). Mörner (6) obtained 1.2 per cent cystine on hydrolysis of a commercial preparation of dried serum proteins and a like amount from purified serum albumin. Inasmuch as the quantitative methods for the isolation of cystine are very inadequate, these figures are presumably much too low.

Analyses of casein by the Van Slyke method for the distribution of nitrogen have yielded results at variance with one another. The analyses of Van Slyke (8) show 0.20 per cent of the total nitrogen as cystine nitrogen, corresponding to a cystine content of about 0.27 per cent. Hart and Sure obtained similar figures (9). On the other hand, Crowther and Raistrick (10) obtained an average of 1.30 per cent[1] of the total nitrogen present as cystine nitrogen, corresponding to 1.77 per cent cystine. Hartley (11) found 2.75 per cent cystine nitrogen, in percentage of total nitrogen, in the serum proteins of ox blood. This was equivalent to 3.78 per cent of cystine.

EXPERIMENTAL.

The methods and general plan of the experiments were similar to those outlined in a former paper (5). The relative efficiency in nutrition of serum albumin, casein, and casein plus cystine, under conditions of a low plane of protein intake, was determined by a comparison of the nitrogen balances of the various experimental periods. In the preliminary experiments, an attempt was made to utilize the pure proteins as the sole source of nitrogen, but the animals refused the food within a short time. In the studies recorded in Tables I to III, therefore, 15 to 20 gm. of beef heart (0.4 to 0.6 gm. of N) were included in the standard diets and the pure proteins added to this. After this change little difficulty was experienced in obtaining complete consumption of the daily ration. The nitrogen added in the form of the beef heart comprised about 20 to 25 per cent of the total nitrogen of the diet, and was constant throughout the experiment, the greater part of the nitrogen being furnished by the protein under investigation.

The casein was prepared according to the method of Hammarsten. The serum protein (Albumin aus Blut, Kahlbaum) was entirely soluble in water, but contained some protein precipitable

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[1] Professor H. S. Grindley of the Division of Animal Nutrition of this University has informed the writer that the work in his laboratory on the analysis of casein by the Van Slyke method has given results similar to those obtained by Crowther and Raistrick (10).

TABLE I.

Dog A, Long Haired Female.

Standard diet: Lard, 50 gm.; cane sugar, 60 gm.; starch, 20 gm.; beef heart, 15 gm.; calcium phosphate, 10 gm.; water, 400 cc.

Period.	Day.	Weight.	Intake nitrogen.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	
I	1	11.16	1.81	2.13	0.27	−0.59	Standard diet and 10 gm. of casein.
	2	11.13	1.81	2.34	0.27	−0.80	
	3	11.13	1.81	2.27	0.27	−0.73	
	4	11.13	1.81	2.24	0.27	−0.70	
	5	11.09	1.81	2.28	0.27	−0.74	
	6	11.09	1.81	2.15	0.27	−0.61	
Total			10.86	13.41	1.62	−4.17	
Average			1.81	2.24	0.27	−0.70	
II	7	11.11	1.81	1.80	0.40	−0.39	Standard diet and 11.65 gm. of serum albumin.
	8	11.11	1.81	1.67	0.40	−0.26	
	9	11.11	1.81	1.72	0.40	−0.31	
	10	11.11	1.81	1.73	0.40	−0.32	
	11	11.09	1.81	1.72	0.40	−0.31	
	12	11.09	1.81	1.72	0.40	−0.31	
Total			10.86	10.36	2.40	−1.90	
Average			1.81	1.73	0.40	−0.32	
III	13	11.07	1.81	2.14	0.37	−0.70	Standard diet and 10 gm. of casein.
	14	11.09	1.81	2.26	0.37	−0.82	
	15	11.07	1.81	2.32	0.37	−0.88	
	16	11.07	1.81	2.28	0.37	−0.84	
	17	11.05	1.81	2.28	0.37	−0.84	
	18	11.05	1.81	2.31	0.37	−0.87	
Total			10.86	13.59	2.22	−4.95	
Average			1.81	2.27	0.37	−0.83	
IV	19	11.09	1.81	1.45	0.30	+0.06	Standard diet, 9.36 gm. of casein, and 0.75 gm. of cystine.
	20	11.11	1.81	0.95	0.30	+0.56	
	21	11.13	1.81	1.13	0.30	+0.38	
	22	11.16	1.81	1.13	0.30	+0.38	
	23	11.16	1.81	1.23	0.30	+0.28	
	24	11.13	1.81	1.20	0.30	+0.31	
Total			10.86	7.09	1.80	+1.97	
Average			1.81	1.18	0.30	+0.33	

TABLE II

Dog G, Long Haired Female Collie.

Standard diet: Lard, 50 gm.; starch, 20 gm.; cane sugar, 70 gm.; beef heart, 15 gm.; bone ash, 10 gm.; water, 450 cc.

Period.	Day.	Weight.	Intake nitrogen.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	
I	1	13.30	1.77	3.13	0.32	−1.68	Standard diet and 10 gm. of casein.
	2	13.23	1.77	2.91	0.32	−1.46	
	3	13.25	1.77	2.67	0.32	−1.22	
	4	13.32	1.77	2.74	0.32	−1.29	
	5	13.32	1.77	2.68	0.32	−1.23	
	6	13.10	1.77	2.65	0.32	−1.20	
Total			10.62	16.78	1.92	−8.08	
Average			1.77	2.80	0.32	−1.35	
II	7	13.20	1.77	1.78	0.25	−0.26	Standard diet, 9.51 gm. of casein, and 0.75 gm. of cystine.
	8	13.20	1.77	1.36	0.25	+0.16	
	9	13.20	1.77	1.42	0.25	+0.10	
	10	13.17	1.77	1.57	0.25	−0.05	
	11	13.18	1.77	1.62	0.25	−0 10	
	12	13.18	1.77	1.76	0.25	−0.24	
Total			10.62	9.51	1.50	−0.39	
Average			1.77	1.59	0.25	−0.07	
III	13	13.16	1.77	2.40	0.21	−0.84	Standard diet, 9.51 gm. of casein, and 0.47 gm. of glycocoll.
	14	13.16	1.77	2.82	0.21	−1.26	
	15	13.14	1.77	2.84	0.21	−1.28	
	16	13.11	1.77	2.57	0.21	−1.01	
	17	13.11	1.77	2.57	0.21	−1.01	
	18	13.07	1.77	2.36	0.21	−0.80	
Total			10.62	15.56	1.26	−6.20	
Average			1.77	2.59	0.21	−1.03	
IV	19	13.07	1.77	2.43	0.34	−1.00	Standard diet, 9.15 gm. of casein, and 1.13 gm. of tyrosine.
	20	13.05	1.77	2.40	0.34	−0.97	
	21	13.00	1.77	2.29	0.34	−0.86	
	22	13.05	1.77	2.32	0.34	−0.89	
Total			7.08	9.44	1.36	−3.72	
Average			1.77	2.36	0.34	−0.93	
V	23	13.00	1.77	2.05	0.21	−0.49	Standard diet, 9.35 gm. of casein, and 0.75 gm. of cystine.
	24	13.11	1.77	1.36	0.21	+0.20	
	25	13.16	1.77	0.99	0.21	+0.57	
	26	13.16	1.77	1.09	0.21	+0.47	
	27	13.19	1.77	1.43	0.21	+0.13	
Total			8.85	6.92	1.05	+0.88	
Average			1.77	1.38	0.21	+0.18	

TABLE III.

Dog A, Long Haired Female; Weight 12.0 Kg.

Standard diet: Lard, 50 gm.; starch, 40 gm.; cane sugar, 60 gm.; beef heart, 20 gm.; calcium phosphate, 10 gm.; water, 400 cc.

Period.	Day.	Intake nitrogen.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		gm.	*gm.*	*gm.*	*gm.*	
I	1	2.04	2.14	0.30	−0.40	Standard diet and 10 gm. of casein.
	2	2.04	2.30	0.30	−0.56	
	3	2.04	2.25	0.30	−0.51	
	4	2.04	2.26	0.30	−0.52	
	5	2.04	2.29	0.30	−0.55	
	6	2.04	2.31	0.30	−0.57	
	7	2.04	2.22	0.30	−0.48	
Total		14.28	15.77	2.10	−3.59	
Average ..		2.04	2.25	0.30	−0.51	
II	8	2.04	1.70	0.32	+0.02	Standard diet and 12.4 gm. of serum albumin.
	9	2.04	1.51	0.32	+0.21	
	10	2.04	1.53	0.32	+0.19	
	11	2.04	1.58	0.32	+0.14	
	12	2.04	1.52	0.32	+0.20	
	13	2.04	1.83	0.32	−0.11	
	14	2.04	1.75	0.32	−0.03	
Total		14.28	11.42	2.24	+0.62	
Average ..		2.04	1.63	0.32	+0.09	
III	15	2.04	1.92	0.34	−0.22	Standard diet and 10 gm. of casein.
	16	2.04	2.21	0.34	−0.51	
	17	2.04	2.07	0.34	−0.37	
	18	2.04	2.13	0.34	−0.43	
	19	2.04	2.09	0.34	−0.39	
	20	2.04	1.92	0.34	−0.22	
	21	2.04	1.94	0.34	−0.24	
Total.		14.28	14.28	2.38	−2.38	
Average ..		2.04	2.04	0.34	−0.34	
IV	22	2.13	1.52	0.28	+0.33	Standard diet, 10 gm. of casein, and 0.75 gm. of cystine.
	23	2.13	1.31	0.28	+0.54	
	24	2.13	1.39	0.28	+0.46	
	25	2.13	1.36	0.28	+0.49	
	26	2.13	1.37	0.28	+0.48	
	27*	2.13	1.51	0.28	+0.34	
	28*	2.13	1.68	0.28	+0.17	
Total		14.91	10.14	1.96	+2.81	
Average ..		2.13	1.45	0.28	+0.40	

* Small amount of food not eaten.

by saturation with NaCl in neutral reaction, as well as some inorganic salts. In a later experiment (not recorded in this paper) in connection with other experiments, the protein was dissolved in water, coagulated by heat, and washed with hot water until tests for chlorides in the wash water were negative. The coagulated protein was then dried *in vacuo* and ground to a fine powder. The results with this more highly purified preparation were similar in every respect to those recorded here for the uncoagulated protein.

As shown in Tables I and III, maintenance of nitrogenous equilibrium was possible under conditions of a lower protein intake when the serum proteins, relatively high in cystine content, were the main source of the protein of the diet than when casein, low in its content of cystine, furnished the bulk of the protein. Thus on a nitrogen intake of 1.81 gm. daily (Table I), the average nitrogen balances were −0.70 and −0.83 gm. respectively during the fore and after periods when casein was fed, as compared with a balance of −0.32 gm. in the serum protein period. With a higher nitrogen intake of 2.04 gm. per day (Table III), the average daily nitrogen balances were −0.51 and −0.34 gm. respectively with casein, and +0.09 gm. with serum albumin. The serum proteins were also more efficient in the maintenance of nitrogenous equilibrium than were the proteins of beef heart when the latter constituted the sole source of protein in the diet (5).

When casein was supplemented by the addition of cystine to the diet (Table I, Period IV; Table II, Periods II and V; Table III, Period IV), the balance of nitrogen became positive in every case except one. The failure of glycocoll and tyrosine to influence the balance of nitrogen (Table II) is additional proof of the specific demand for cystine on a diet poor in this amino-acid. These results are in harmony with the work of Osborne and Mendel (1), in which these observers reported that growth in white rats occurred at a lower plane of protein intake when casein was supplemented by cystine than with casein alone. In the present series of experiments, casein supplemented by cystine was apparently somewhat superior in nutritive value to serum albumin. This may possibly be explained by the fact that some amino-acid other than cystine is the limiting factor for this protein.

SUMMARY.

The relative efficiencies in nutrition of casein, a protein low in cystine content, and of serum albumin, high in cystine content, have been studied in dogs. Serum albumin under conditions of a low protein intake is more effective in maintaining nitrogenous equilibrium than is casein. When casein is supplemented by cystine, however, it is as efficient for the maintenance of nitrogenous equilibrium as is serum albumin. These experiments furnish additional evidence that cystine is essential for maintenance as well as growth.

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A METHOD FOR THE DETERMINATION OF METHEMOGLOBIN AND HEMOGLOBIN IN BLOOD.

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While studying a type of cyanosis due to change in the blood pigment, with the formation of methemoglobin, need arose for a simple and accurate quantitative method for methemoglobin. A further incentive was the belief that such a method would be of considerable value in hospital or clinical laboratories, for application in cases of severe methemoglobinemia, when the amount of methemoglobin in the blood is of value in determining the advisability of certain therapeutic procedures, such as transfusion.

The method to be described was suggested by the observation that in blood containing methemoglobin the hemoglobin determined colorimetrically was considerably greater than the hemoglobin calculated from the oxygen capacity.[1]

Stadie[2] has recently published a method for methemoglobin identical with ours except that the total hemoglobin is determined as cyanhemoglobin after converting the hemoglobin into methemoglobin.

Principle of the Method.

The method is based upon the fact that while oxygen, as is well known, is liberated from hemoglobin by potassium ferricyanide it is not liberated from methemoglobin even *in vacuo*. The total hemoglobin (hemoglobin + methemoglobin) is determined colorimetrically as methemoglobin, using potassium ferricyanide to convert the hemoglobin into methemoglobin. The oxygen capacity is determined by Van Slyke's method,[3] from which is calculated the hemoglobin only. The hemoglobin as calculated from the oxygen capacity is subtracted from the total hemoglobin determined colorimetrically as methemoglobin. The difference is the amount of methemoglobin.

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[1] A preliminary note was published by McEllroy, W. S., *J. Am. Med. Assn.*, 1919, lxxiii, 1927. The method was reported before the American Society of Biological Chemists (McEllroy, W. S., *J. Biol. Chem.*, 1920, xli, p. xlvii).

[2] Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237.

Description of the Method.

The blood is drawn from an arm vein and oxalate used to prevent clotting. 1 cc. of the blood is transferred to a 50 cc. volumetric flask containing 20 cc. of distilled water. For this purpose an Ostwald pipette calibrated to contain 1 cc. is satisfactory. The blood is drawn up to the mark, allowed to drain into the flask, and the drop in the tip blown out. Distilled water is then drawn up into the pipette and discharged into the flask. This is repeated until the blood in the pipette and any adhering to the neck of the flask is washed down into the flask. 1 cc. of a 4 per cent solution of potassium ferricyanide is added and the flask agitated to insure mixing. After standing for 5 minutes it is diluted to the mark with water, mixed, and compared in the colorimeter with a standard solution of methemoglobin. The cup containing the standard is set at 10 or 15 mm. The result gives the total hemoglobin (hemoglobin + methemoglobin) in gm. per 100 cc. of blood.

The oxygen capacity is determined on a portion of the same blood. From this is calculated the hemoglobin content in gm. per 100 cc. The hemoglobin calculated from the oxygen capacity is subtracted from the total hemoglobin (hemoglobin + methemoglobin) and the difference is the amount of methemoglobin in gm. per 100 cc. of blood.

Preparation of the Standard.

The standard solution of methemoglobin having a known hemoglobin content is prepared from normal defibrinated or oxalated blood. Defibrinated ox blood is convenient. The hemoglobin content of the normal blood is determined from its oxygen capacity. 10 cc. of the blood are transferred to a 500 cc. volumetric flask containing 200 cc. of water and 10 cc. of a 4 per cent solution of potassium ferricyanide solution added. The flask is agitated and allowed to stand for 10 minutes. It is then diluted to the mark with water and mixed. This gives a standard solution of methemoglobin, the blood pigment value of which is known, for the determination of the total hemoglobin as described above. In case the standard is much stronger than the unknown, suitable dilution of the standard should be made until one approximating in concentration that of the unknown is obtained. Of course the degree of dilution of the standard must be known.

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[3] Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.

The method of calculation is best illustrated by means of an example.

Standard solution of methemoglobin = 18 gm. of hemoglobin per 100 cc. of blood.

Colorimetric determination of total pigment as methemoglobin = standard 10 mm. and unknown 11 mm.

Then $\frac{10}{11} \times 18 = 16.36$ gm. of total pigment per 100 cc.

Hemoglobin calculated from oxygen capacity = 15 gm. per 100 cc.

Therefore $16.36 - 15 = 1.36$ gm. of methemoglobin per 100 cc. of blood.

DISCUSSION.

The total hemoglobin (hemoglobin + methemoglobin) cannot be determined colorimetrically as carbon monoxide hemoglobin because the methemoglobin is not converted into carbon monoxide hemoglobin quantitatively. The methemoglobin, however, apparently may be converted into acid hematin by hydrochloric acid and the total hemoglobin determined as such. As the spectroscopic data on this point is still incomplete the results will be published later.

The color of the methemoglobin is very nearly identical with that of acid hematin and can be compared accurately colorimetrically. Although the methemoglobin and acid hematin approximately match in color they have very dissimilar colorimetric values.

CONCLUSIONS.

In blood containing methemoglobin the amount of methemoglobin and hemoglobin can be determined quickly and accurately by the method described.

VITAMINE STUDIES.

V. THE ANTISCORBUTIC PROPERTIES OF RAW BEEF.*

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(Received for publication, March 30, 1920.)

INTRODUCTION.

The rapidly accumulating literature, both in England and in this country, concerning the antiscorbutic properties of food materials lends considerable support to the view that we are dealing with a third "food hormone" which can be placed in the list of substances called vitamines. In fact, the recognition of this vitamine has become sufficiently general for Drummond,[1] following the nomenclature of McCollum and Kennedy,[2] to designate this vitamine as "water-soluble C."

The work described in this paper was initiated as a part of a larger cooperative problem under investigation at this institution; *i.e.*, the study of the effect of heat as employed in the ordinary methods of food preparation upon the vitamine content of some of our common food materials. Meat was chosen as the first food to be studied, owing to the fact that human experience had indicated that this food possessed antiscorbutic properties, and also because our facilities were unusually good for obtaining meats cooked in a variety of ways.

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A preliminary statement of this work was published in *Science*, 1919, l, 184.

[1] Drummond, J. C., *Lancet*, 1918, ii, 482.

[2] McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 493.

Our findings with regard to the antiscorbutic properties of beef, which are described in this paper, were such that the original plan, involving the cooking of beef, was, of necessity, abandoned.

Lind[3] is the first observer, as far as we are aware, to ascribe antiscorbutic properties to meat. In his early studies of tropical diseases (1771) this writer made the interesting observation that a soup prepared from the flesh of the green turtle was curative in human scurvy.

About 120 years later Barlow[4] advocated the use of meat juice or meat gravy as an antiscorbutic food for infants, although he emphasized the superiority of uncooked meat over that which had been cooked.

Smith,[5] in an interesting article on the historical phases of antiscorbutic foods and drinks, cites the case of the Grinnell expeditions in which "a certain amount of fresh meat" failed to prevent scurvy on the first expedition, while scurvy was thought to have been prevented on the second expedition by occasional supplies of fresh meat.

More recently Stefánsson,[6] describing his experiences in the Arctic regions, states that fresh meat prevented and cured scurvy in his party.

Wiltshire,[7] on the other hand, reports observations on 132 cases of scurvy among Serbian soldiers whose daily diet had contained meat which had been frozen. This writer is of the opinion that fresh raw meat contains "a small amount of antiscorbutic vitamine" but that freezing and cooking "render meat practically useless."

Chick, Hume, and Skelton[8] were unable to note any beneficial effect when meat juice was fed to guinea pigs, while Pitz[9] is of the opinion that dried meat delayed the onset of scurvy symptoms in the same type of experimental animal, due to the better plane of protein intake. This work is open to criticism, however, due to the fact that milk was fed *ad libitum*. The antiscorbutic property of milk is undoubtedly proportional to the quantity ingested and also, as we[10] suggested in a previous paper, to the nature and quantity of the fresh feeds eaten by the cow.

In a recent publication Givens and McClugage[11] have substantiated our work showing that they were unable to prevent scurvy in guinea pigs and, furthermore, they found that it was impossible to prolong life on scorbutic diets by the ingestion of dehydrated beef. These results are not in accord with those of Pitz,[9] the explanation of which, Givens and McClugage believe, lies in the amount of milk consumed by the guinea pigs in Pitz's experiment. This view is also in accord with our former statements;[10] that is, that the milk should always be fed quantitatively, for "the antiscorbutic properties of milk are proportional to the amount of milk ingested."

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[3] Lind, J., An essay on diseases incidental to Europeans in hot climates, 1771, 2nd edition, 206.

[4] Barlow, T., *Lancet*, 1894, ii, 1075.

[5] Smith, A. H., *Lancet*, 1918, ii, 813.

[6] Stefánsson, V., *J. Am. Med. Assn.*, 1918, lxxi, 1715.

[7] Wiltshire, H. W., *Lancet*, 1918, ii, 811.

[8] Chick, H., Hume, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131.

[9] Pitz, W., *J. Biol. Chem.*, 1918, xxxvi, 439.

[10] Dutcher, R. A., Pierson, E. M., and Biester, A., *Science*, 1919, l, 184.

[11] Givens, M. H., and McClugage, H. B., *Science*, 1920, li, 273.

EXPERIMENTAL.

Care of Animals.—The animals used in the experiments described in this paper were healthy young guinea pigs weighing between 150 and 350 gm. No guinea pigs were used until they had been under observation for several days during which they were fed a normal diet of oats, cabbage, carrots, and hay. Guinea pigs which did not grow normally were eliminated from the experiment. The animals were grouped according to weight, each experimental group being made up of one heavy guinea pig, one light guinea pig, and two which fell between the two extremes in regard to body weight. This was done in order that the groups should be comparable, one with the other, and to eliminate as far as possible the effect of age and size of the animals on the average length of time elapsing before the onset of the disease.

The animals were confined, singly, in cages containing removable metal trays which were cleaned once or twice a week and sprayed with a cresol preparation. Clean fresh sawdust was used as bedding to absorb moisture. Weight records were taken every 4 days before feeding and the animals were under observation at practically all hours of the day. Autopsies were performed in all cases soon after death and ankle joints and costochondral junctions were placed in Zenker's fluid, washed and carried through dilute alcohol, and finally preserved in 95 per cent alcohol for histological examination.

Beef Extract.—Lean beef freed from gristle and fat was finely ground, and weighed portions were mixed with water and allowed to stand in a cool place for 12 to 15 hours. The mixture was then subjected to pressure in a hand press and the liquid extract carefully removed. The dry meat pulp was then treated with water a second time and allowed to stand for 2 hours. After a second pressing a third extraction was made. The water extracts were combined and made to such a volume that 2 cc. represented the water-extractable material from 1 gm. of beef. Fresh extracts were made every 3rd day and these were kept on ice until used. The beef was procured in the open market.

Experimental Diet.—As has been customary with many investigators in this field, oats were fed *ad libitum*. In order to improve the protein and salt content of the ration 25 cc. of autoclaved milk were given daily. The milk was autoclaved for 1 hour at 120°C. to eliminate any possibility of introducing the antiscorbutic vitamine in the milk. Little difficulty was experienced in getting the animals to take the milk quantitatively from dishes, provided water was withheld for a time before feeding. The beef extract was fed by hand by means of a large medicine dropper. In a few cases it was also necessary to feed the milk by hand, especially during the latter stages of the disease.

The groups of animals in Series I received the following diets:

Group I. Basal diet (oats, water, 25 cc. of autoclaved milk).
" II. " " + 10 cc. of beef extract.
" III. " " + 20 " " " "
" IV. " " + 30 " " " "
" V. " " + 40 " " " "
" VI. " " + 5 gm. " chopped raw beef.
" VII. Oats and water only.

The detailed results of the experimental work in Series I are shown in Table I.

Examination of the data in Table I shows that the presence of the meat extract had little effect in preventing the onset of scurvy symptoms or in delaying death from scurvy. When plotted, all the curves are of the same type irrespective of the amount of beef extract fed. The average length of time elapsing before the symptoms were noted was about 15 days, which is about the time usually expected on oats alone. The average length of life was about 26 days, which is also the time usually expected on a scorbutic type of diet. It was not considered necessary to feed the dry beef residues which remained after extraction for the reason that the water-soluble antiscorbutic substance could not have remained behind in appreciable quantities in view of the methods employed. The feeding experiment with the solid raw beef (Table I) substantiates our original opinion in this regard, for the ingestion of the chopped raw beef showed no beneficial effects.

Histological examinations were made of joints and costochondral junctions of representative animals in each group and, in every case, our clinical observations were substantiated by the histological findings. In general, our observations are in accord with other investigators in this field with regard to the symptomatology of scurvy in guinea pigs. We have felt that the face ache position has been somewhat overemphasized as far as symptomatology is concerned. To be sure we have noted it in a number of cases, but a large proportion of our scorbutic animals have never been found in this position. On the other hand, it is not uncommon to find normal guinea pigs sleeping in a manner which simulates the face ache position. Other symptoms, such as partial paralysis of the hind quarters, tenderness of joints, swelling of joints, etc., have been observed to occur with great regularity and it does not require much experience to be able to tell within a reasonable limit of error when the first symptoms of scurvy appear. We have also found, in addition, swollen joints (which harden into exostoses), fragility of bones, and loosening of teeth. Postmortem examination invariably reveals hemorrhagic conditions and extravasations in many of the tissues, especially in the muscles of the hind legs. Swollen costochondral junctions are usually present and these are often hemorrhagic.

At the termination of the experiments outlined in Table I we were forced to conclude that beef extract does not contain a water-soluble antiscorbutic substance in sufficient quantity to prevent scurvy in the guinea pig. We realized that meat extractives and milk were not normal ingredients of a guinea pig diet and it was felt that it was possible that these foods might possess properties which were actually deleterious; therefore the work was repeated feeding orange juice in the presence and in the absence of meat extract.

In the confirmatory work (Table II, Series II), the basal diet was identical with that of Series I with the exception that 20 cc. of pasteurized milk were fed instead of 25 cc. of autoclaved milk. This was done with the view of dispensing with the work of autoclaving the milk, for it is well known[12] that milk is so deficient in the antiscorbutic vitamine that the small quantity fed (20 cc.)

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[12] Barnes, R. E., and Hume, E. M., *Lancet*, 1919, ii, 323.

TABLE I.

Series I.

Influence of Cold Water Extract of Raw Beef upon Scurvy in Guinea Pigs (2 Cc. from 1 Gm. of Beef). Basal Diet Consisted of Oats and Water ad Libitum and 25 Cc. of Autoclaved Milk.

Group and diet.	Animal No.	Initial weight.	Final weight.	Gain or loss.	Day on which first scurvy symptoms were noted.	Average time for group.	Length of life.	Average life for group.	Postmortem examination.	Remarks.
		gm.	*gm.*	*per cent*		*days*	*days*	*days*		
I Basal diet.	1	304	201	−33.9	10	11.7	19	21.7	Scurvy.	
	2	225	150	−33.3	7		23		"	
	3	280	160	−42.9	15		20		"	
	4	160	120	−25.0	15		25		"	
II Basal diet + 10 cc. beef extract.	5	358	250	−30.2	23	18.2	28	25.2	" and pneumonia.	
	6	223	210	− 5.8	13		23		"	
	7	253	160	−36.8	22		25		"	
	8	189	160	−15.3	15		25		"	
III Basal diet + 20 cc. beef extract.	9	345	240	−30.4	14	14.2	23	24.0	"	
	10	240	157	−34.5	17		26		"	
	11	211	170	−19.5	13		25		"	
	12	174	130	−25.3	13		22		"	
IV Basal diet + 30 cc. beef extract.	13	257	183	−28.8	15	14.6	25	25.6	" and pneumonia.	
	14	330	245	−25.8	15		31		"	
	15	226	167	−26.1	14		21		"	

V Basal diet + 40 cc. beef extract.	17	365	248	−32.1		18.6	30	26.0	Scurvy.	
	18	278	195	−29.9	17		25		"	
	19	233	200	−14.2	13		22		" and pneumonia.	
	20	153	165	+ 7.8	26		27		"	Cured on orange juice; no histological evidence of scurvy.
VI Oats + 5 gm. chopped raw beef.	51	320	175	−45.3		20(?)	22	26.0	General inanition.	
	52	325	225	−31.8			24		Scurvy.	
	53	250	147	−41.2	20		30		General inanition.	No scurvy lesions.
	54	271	180	−34.6			28		Scurvy.	
VII Oats and water.	21	247	135	−45.3	20	17.5	25	26.0	"	
	22	183	120	−34.4	15		24		"	
	23	175	110	−37.1			29		"	

could not influence the course of the disease to an appreciable extent.

The diets fed in Series II were as follows:

Group VIII. Basal diet (oats, water, 20 cc. of pasteurized milk).
" IX. " " + 5 cc. of orange juice.
" X. " " + 5 " " " " + 10 cc. of meat extract.
" XI. " " + 10 " meat extract.

The details of the feeding experiment in Series II are summarized in Table II.

The data in Table II verify the results obtained in Series I; *i.e.*, that the presence of meat extract did not prevent the onset of scurvy. When orange juice was fed (Group IX) no scurvy developed, and the same is true for Group X which received orange juice and beef extract. When the orange juice was omitted (Group XI), the presence of meat extract had no effect so far as scurvy is concerned. It would appear, also, that the beef extract had no detrimental effect when fed in the presence of orange juice.

The length of life, in Series II, was prolonged beyond the usual period, the probable explanation depending upon the diet of the cow from which the milk was obtained rather than upon the presence of the meat extract or the fact that the milk was not autoclaved. When the same milk was autoclaved for 1 hour at 120°C. curves and data were obtained similar to those with the pasteurized milk, thereby eliminating the possibility of the presence of antiscorbutic vitamine in the milk. Our reasons for this view will be published in detail in a later paper.

It may be possible that the explanation for the antiscorbutic properties of the meat in the diet of Stefánsson's party[6] lies in the fact that the meat was obtained, almost entirely, from the seal and the bear and included not only muscle tissue but other tissues, such as the liver. It is well to emphasize at this point that too great care cannot be exercised in applying the results of experiments with guinea pigs and rats to problems in human nutrition. Recent work of Hess and Unger[13] indicates that the demand of infants for the fat-soluble vitamine is much less than

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[13] Hess, A. F., and Unger, L. J., *J. Am. Med. Assn.*, 1920, lxxiv, 217.

TABLE II.

Series II.

Confirmatory Data Relative to the Influence of Beef Extract in the Presence of Orange Juice. Basal Diet Consisted of Oats and Water ad Libitum + 20 Cc. of Pasteurized Milk.

Group and diet.	Animal No.	Initial weight.	Final weight.	Gain or loss.	Day on which first scurvy symptoms were noted.	Average time for group.	Length of life.	Average life for group.	Postmortem examination.	Remarks.
		gm.	*gm.*	*per cent*		*days*	*days*	*days*		
VIII Basal diet.	24	311	220	−29.3	29	24.6		39.5		Changed to curative diet on 40th day.
	25	286	180	−37.1	26		40		Scurvy.	
	26	265	205	−22.6	19		39		"	
IX Basal diet + 5 cc. orange juice.	28	365	360	− 1.4						Changed to another diet on 60th day.
	29	367	435	+18.5						" " " " " 60th "
	30	320	393	+22.8						" " " " " 60th "
	31	230	245	+ 6.5						" " " " " 60th "
X Basal diet + 5 cc. orange juice + 10 cc. beef extract.	32	265	333	+25.7						" " " " " 60th "
	33	235	360	+53.2						" " " " " 60th "
	34	283	380	+34.3						" " " " " 60th "
	35	312	305	− 2.2						" " " " " 60th "
XI Basal diet + 10 cc. beef extract.	37	295	294	− 0.3	25	31.5		54.6	Scurvy.	Choked to death on 28th day.
	38	280	175	−37.5	36		54		"	
	39	268	199	−25.7	29		41		"	
	40	425	260	−38.8	36		69			Changed to another diet on 69th day.

that indicated by previous work with rats. Similar discrepancies will doubtless appear as vitamine work progresses, due to the varying physiological demands for specific vitamines by the different species of animals.

SUMMARY AND CONCLUSIONS.

Guinea pigs were fed diets of oats, water, and an amount of milk sufficient to improve the diet but insufficient to prevent scurvy. These animals developed scurvy and died. When water extracts of raw lean beef were fed representing 5, 10, 15, and 20 gm. of raw beef no differences could be noted in the time of onset of scurvy or in the length of life of the experimental animals. Orange juice, added to the basal diet, prevented scurvy, both in the presence and absence of meat extract. The excellent condition of the animals on the orange juice-beef extract diet shows conclusively that the poor condition of the animals on the beef extract diet was due to the absence of the antiscorbutic vitamine rather than to any deleterious property of the beef extract.

In conclusion, we wish to acknowledge the assistance of Dr. H. E. Robertson for his preparation and interpretation of some of our histological material, Dr. C. P. Fitch for assistance in some of the postmortem examinations, and Mr. O. Mydland for assistance in caring for the experimental animals.

CRITICAL STUDY OF METHODS FOR THE DETECTION OF METHYL ALCOHOL.

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During the years 1918 and 1919, I have had occasion to examine over 700 human organs for alcohol. In addition to this, about 250 liquors of various descriptions were analyzed.

In the work with liquors, wines, whiskeys, and cordials, a very interesting and far reaching observation was made, particularly in connection with medicolegal work. It was found, when the liquors were tested in the customary manner, that is by oxidation of the alcohols to aldehydes and then applying the usual color reactions (milk-hydrochloric acid test, resorcin test, gallic acid test), that many liquors which contained no methyl alcohol at all gave very good positive reactions to one or all three of the above named tests. Then again, liquors which contained even as much as 10 per cent methyl alcohol would often give a milk-hydrochloric acid test no different from a good ethyl alcohol liquor; that is, the characteristic methyl alcohol reaction was entirely masked (28). The cause of these false and masked reactions was the presence in the oxidized products of the liquors of a number of substances such as acetone, acetaldehyde, ethyl alcohol, formic acid, dimethyl-ethyl-carbinol, secondary and tertiary butyl alcohol, essential oils, acrolein, and furfurol. The more examinations I made, the more I was impressed with the unreliability of the above tests. Therefore, it appeared of great importance to make a study of all the tests for methyl alcohol reported in the literature in order to find a set of tests which, when properly applied, would give results upon which there could be placed absolute reliance.

58 tests for methyl alcohol were found in the literature. All these were put through a critical study upon eighteen liquors of the following description:

Eight typical good grain alcohol whiskies.

One combined distillate of 58 liquors free from methyl alcohol and containing 42 per cent alcohol.[1]

One mixture of 5 per cent methyl alcohol plus 55 per cent of above combined distillate plus 40 per cent water.

One mixture of 10 per cent methyl alcohol plus 50 per cent of above combined distillate plus 40 per cent water.

One mixture of 20 per cent methyl alcohol plus 40 per cent of above combined distillate plus 40 per cent water.

One mixture of 30 per cent methyl alcohol plus 30 per cent of above combined distillate plus 40 per cent water.

Five typical methyl alcohol liquors (confiscated).

Each of these eighteen liquors was subjected to all the 58 tests. The reactions marked with an asterisk were not completely studied, either because the reagent was not obtainable or because the small amount on hand was not sufficient to permit the testing of all the eighteen samples. By applying each test to a series of liquors, as the one indicated above, practically all the characteristics, such as sensitiveness, reliablity, specificity, and influence upon the reaction of foreign substances, were brought out.

The tests for methyl alcohol can be classified into two groups: (*A*) That group in which the methyl alcohol must first be oxidized into formaldehyde before the tests are applied; (*B*) that group in which no oxidation is necessary, the test being applied directly to the alcohol molecule. In both groups of methods it is imperative, first, to distill fractionally the liquor, the object being to free the alcohols from non-volatile admixed substances[2] and, second, to obtain the alcohol in more concentrated form.

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[1] The object of using the combined sample of so many liquors was to get as many as possible of the interfering substances that naturally occur in the average drinks.

[2] Some ciders, when oxidized directly, responded beautifully to all the color reactions for formaldehyde. If they were previously distilled and then oxidized, however, no such reactions were obtained. The physical tests and the tests depending on crystalline structure proved the absence of methyl alcohol.

Methods of Group A.

In this group the tests depend upon the reaction of formaldehyde with the various reagents mentioned below. It is therefore necessary first to convert the methyl alcohol to formaldehyde. This is brought about by oxidation. Several methods for oxidizing methyl alcohol into formaldehyde are available:

(*a*) Plunging a red hot copper spiral into the liquid.

(*b*) Passing the vapors of alcohol mixed with air through a tube over hot copper.

(*c*) By finely divided platinum.

(*d*) Alkaline permanganate.

(*e*) Potassium dichromate and sulfuric acid.

(*f*) Chromic acid.

(*g*) Sodium persulfate and sulfuric acid.

(*h*) Manganese peroxide and sulfuric acid.

(*i*) Lead peroxide and acid or alkali.

(*j*) Hydrogen peroxide and alkali.

All these methods have been tried and I prefer the oxidation with dichromate or permanganate. One must not forget that during oxidation products other than aldehyde are formed. There is always a large amount of acid produced, because it is impossible to regulate the reaction so that the oxidation produces aldehyde and goes no farther. The best we can do is to adjust our conditions so as to give the maximum amount of aldehyde and the minimum amount of acid. For this no fixed rule can be given, as it depends upon the ratio of alcohol present to that of the oxidizing agent, and also upon the temperature. At this stage it is well to point out that in these oxidations there is always a trace of formaldehyde formed from ethyl alcohol. This, however, is not important because it is a very small fraction of 1 per cent and yields reactions so faint, compared to even a 0.5 per cent methyl alcohol liquor, that it cannot be mistaken. The conditions best suited for a 40 to 45 per cent (80 to 90 proof) alcoholic beverage are shown below in the "Typical procedure for methyl alcohol detection." In liquors containing less alcohol, the oxidizing agent should be correspondingly decreased.

After the oxidation, it is necessary to destroy the excess of the oxidizing agent. This is done by adding sulfurous acid and boiling until no sulfur dioxide odor is detectable, or by adding oxalic acid and filtering, if necessary, or by adding oxalic acid and distilling. I have found that the last of these three methods is by far the best. In distilling, if both methyl and ethyl were originally present in the liquor, the acetaldehyde comes over in the first 30 cc., and the formaldehyde comes over in the next 60 to 100 cc. This, of course, is not a sharp division, but it separates the major portion of the acetaldehyde from the formaldehyde and is important, as acetaldehyde is an annoying substance in the reactions for formaldehyde. A further advantage is that such distillation leaves behind the residual substances from the oxidizing agent.

The oxidized and fractionally distilled material can then be tested by any of the following reactions.[3]

Formaldehyde with Phenylhydrazines.

1. Phenylhydrazine hydrochloride plus ferric chloride plus concentrated hydrochloric acid yield a beautiful crimson color (1–4). (A)

2. Phenylhydrazine hydrochloride plus ferric chloride and stratifying concentrated sulfuric acid underneath yield a beautiful crimson-colored ring (4, 5). (D)

3. Phenylhydrazine hydrochloride plus sodium nitroprusside plus sodium hydroxide yield a deep blue color (1–4, 6–8). (A)

4. Phenylhydrazine hydrochloride plus potassium ferricyanide plus concentrated hydrochloric acid yield a red color. Too much formaldehyde makes the test less sensitive; therefore the material should be diluted before testing (9, 10). (A)

5. Phenylhydrazine hydrochloride plus sodium acetate plus sulfuric acid and boiling yield a yellowish green color (11, 12). (D)

6. Phenylhydrazine hydrochloride plus manganese chloride plus concentrated hydrochloric acid give a pink color (13). (E)

7. Methylphenylhydrazine (symmetrical) plus hydrochloric acid give a precipitate of dimethyldiphenylhexahydrotetrazine (14, 15). (*)

8. α-α-methylphenylhydrazine plus hydrochloric acid give a dark green color and a precipitate may also form (14). (*)

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[3] The letters after each test have the following significance:
A. Reliable, extremely sensitive, technique fairly simple.
B. “ for 5 per cent or more, “ “ “
C. “ “ 5 “ “ “ “ “ long and tedious.
D. “ in great majority of cases but not in all.
E. Unreliable.
(*) Not completely studied.

9. *p*-Dihydrazinodiphenylhydrochloride and warming to 50–60°C. yield a flocky yellow hydrazone. This test can be used for separating this hydrazone from the hydrazones of other aldehydes (16). (B)

10. *p*-Nitrophenylhydrazine in an acetic acid solution yields yellow needle-shaped crystals, melting at 181–182°C. If this product is made alkaline with sodium hydroxide, a violet color is produced (17). (D)

Formaldehyde with Phenols.

11. β-Naphthol plus hydrochloric acid give needle-shaped crystals, melting at 190°C. Acetaldehyde produces no such crystallization (18). (B)

12. Phenol and stratifying sulfuric acid underneath yield a red ring (19). (D)

13. Resorcin plus sodium hydroxide and heating yield a red color (20). (D)

14. Resorcin and stratifying sulfuric acid underneath yield a red ring (21–29). (D)

15. Resorcin plus hydrochloric acid and boiling yield a red color (30). (D)

16. Dimethylhydroresorcin produces a difficultly soluble condensation product (31). (*)

17. Naphthoresorcin plus hydrochloric acid yield a flocculent precipitate which darkens on standing (32). (*)

18. Phloroglucin plus sodium hydroxide yield a red color (20, 33–41). (D)

19. Phloroglucin plus hydrochloric acid and heating yield a very finely divided precipitate and the solution becomes orange in color (42, 43). (D)

20. Pyrogallol plus sulfuric acid give a chocolate-brown color (44). (E)

21. Pyrocatechol plus ferrous sulfate and stratifying sulfuric acid underneath give a violet ring (45). (E)

22. Guaiacol plus ferrous sulfate and stratifying sulfuric acid underneath give a violet ring (45). (E)

23. Gallic acid and stratifying sulfuric acid underneath give a green ring, changing to a deep blue ring (25, 26, 46, 47). (D)

Formaldehyde with Alkaloids.

24. Morphine (a few mg.) plus concentrated sulfuric acid gradually develop a violet color, changing to indigo blue. This reaction may also be modified to the production of a ring test (48–50). (A)

25. Morphine (a few mg.) plus milk plus concentrated sulfuric acid give a pink color, changing to deep blue. If the acid is stratified below the mixture, a ring of similar color is produced (51). (A)

26. Codeine (a few mg.) plus concentrated sulfuric acid give a violet color (52). (A)

27. Apomorphine (a few mg.) plus sulfuric acid give a violet color. This test is even more specific than the morphine test (53). (A)

Formaldehyde with Proteins.

28. Milk plus ferric chloride plus hydrochloric acid and heating give a violet color (29, 54). (D)

29. Milk and stratifying sulfuric acid underneath give a violet ring (55–57). (D)

30. Milk plus ferric chloride and stratifying concentrated sulfuric acid underneath give a violet ring (58–60). (D)

31. Casein (ammoniacal) solution plus molybdic salt and stratifying sulfuric acid underneath give a violet ring (61). (D)

32. Albumin plus ferric chloride and stratifying concentrated sulfuric acid underneath give a violet ring (62). (D)

33. Albumin plus nitrous acid plus hydrochloric acid and warming give a violet color (63). (A)

34. Peptone plus ferric chloride plus hydrochloric acid and warming give a violet color (64). (A)

Formaldehyde with Amines.

35. Diphenylamine (sulfuric acid solution) gives a green color (65). (B)

36. Dimethylaniline plus sulfuric acid condense with formaldehyde into tetramethyldiaminodiphenylmethane. The latter when oxidized by lead peroxide in an acetic acid solution assumes an intense blue color, becoming more intense on heating. The blue color obtained with acetaldehyde disappears on heating (66–73). (C)

37. Aniline condenses with formaldehyde into anhydroformaldehyde-aniline (74). (E)

38. Carbazol in hot glacial acetic acid and in the presence of a trace of hydrochloric acid or of sulfuric acid gives a white precipitate (75). (D)

39. Carbazol in concentrated sulfuric acid gives a blue color and even a blue-green precipitate (76). (A)

Formaldehyde with Miscellaneous Substances.

40. Benzoyl peroxide plus sulfuric acid give a blood-red color. If much water is added to this, the color disappears (77). (*)

41. Concentrated ammonia condenses with formaldehyde on evaporation into hexamethylenetetramine (urotropin). The latter gives characteristic crystals with mercuric chloride (78). (D)

42. Fuchsin (reduced) plus sulfuric acid gradually develop a violet-red color (79, 80). (A)

43. Oxidizing to carbon dioxide under conditions in which ethyl alcohol is oxidized into acetic acid. The carbon dioxide is then identified by the usual reactions (81, 82). (C)

44. Oxidizing with hydrogen peroxide in a solution made alkaline with sodium hydroxide and performed at low temperature (5° or lower) yields formic acid. The reducing power of this acid is then detected (83). (E)

45. Mercuric oxide plus sodium sulfite in alkaline solution precipitate other aldehydes but do not precipitate formaldehyde (84, 85). (E)

46. Oxidizing to formaldehyde and detecting its odor. This test is usually performed directly on the liquor. Oxidation is performed by plunging a red hot copper spiral into the liquor (86). (E)

From the study of the above set of tests, based on the detection of formaldehyde produced by oxidation from the methyl alcohol, the following reactions are to be preferred: Five color reactions; namely, (No. 1) phenylhydrazine-ferric chloride-hydrochloric acid, (No. 3) phenylhydrazine-sodium nitroprusside-sodium hydroxide, (No. 27) apomorphine-sulfuric acid, (No. 34) peptone-ferric chloride, (No. 42) reduced fuchsin-sulfuric acid; and two crystal-producing tests, (No. 11) β-naphthol-hydrochloric acid, and (No. 41) hexamethylenetetramine-mercuric chloride. The five color tests are extremely sensitive, one part in 200,000 being easily detected. The two crystal-producing tests are for specificity rather than for the detection of minute quantities. When the latter two tests are done in accordance with the foregoing methods, as low as 5 per cent methyl alcohol can be detected. In order to get crystals from material containing less than 5 per cent methyl alcohol, it is necessary first to concentrate by fractional distillation. The structure of the crystals and their melting point are specific tests. The production of crystals is definite and convincing proof that the suspected liquid contains formaldehyde. The technique in detail of these tests is given below.

Methods of Group B.

Within this class fall those methods which are based upon the interaction of the methyl alcohol molecule with the reagent added, no oxidation being necessary. Methyl alcohol is used with the following substances.

1. Sodium alizarin sulfonate in alkaline solution produces a clear violet color with both ethyl and methyl alcohol. If crystals of oxalic acid are

added and the mixture is shaken, methyl alcohol will produce a dirty, slimy precipitate, while with ethyl alcohol the solution will remain clear (87). (D)

2. Sodium salicylate plus concentrated sulfuric acid yield the odor of methyl salicylate (88, 89). (E)

3. Iodine plus red phosphorus produce methyl iodide. This product is then treated with aniline and sodium hydroxide is added. The oily bases which rise to the top are removed and oxidized with a mixture of sand plus sodium chloride plus cupric nitrate and heat. The product is taken up with warm alcohol. It assumes a red color if only ethyl alcohol was present originally, but if methyl alcohol was present (even 1 per cent) the color obtained is violet (methyl aniline violet) (73, 90–94). (C)

4. Iodine plus red phosphorus yield methyl iodide. This is distilled with silver nitrite, yielding nitromethane. To this product are added ammonia and vanillin and then it is heated. If methyl alcohol is present, a red color develops (95). (E)

5. Iodine plus red phosphorus yield methyl iodide. This is then distilled with silver nitrite, yielding nitromethane. To this are added ammonia and sodium nitroprusside. A blue color develops which, on standing, changes to green and then to yellow (96). (E)

6. Iodine plus red phosphorus produce methyl iodide. Separate the methyl iodide from the ethyl iodide (if present) by fractional distillation, and then get the saponification number. As ethyl iodide and methyl iodide have different saponification numbers, the amount of each can be estimated (97). (C)

7. Iodine plus red phosphorus yield methyl iodide and ethyl iodide. Purify these by washing, drying, and redistilling and then take the specific gravity. From the specific gravity the amount of each can be estimated (98). (C)

8. Borax when mixed with methyl alcohol and ignited burns with a green flame (99). (E)

9. Hydroxylamine plus potassium hydroxide and boiling for 7 hours under a reflux condenser yield cyanide. Ethyl alcohol, acetaldehyde, acetal, and amyl alcohol do not yield cyanide under these conditions. The cyanide so produced is detected by the Prussian blue test or the ammonium sulfocyanide test (100). (A)

10. Oxalic acid yields typical crystals on boiling and then cooling. These crystals, if pure, melt at 54°C. (101). (D)

11. Refraction and its relation to the specific gravity is a reliable method of differentiation (54, 102). (B)

12. Selenic acid and a trace of silver bromide will precipitate ethyl but not methyl alcohol (103). (D)

All the methods of this group require a large amount of alcohol. There is none here which can detect very small amounts. The technique is, in the majority of these tests, long and tedious, as, for example, in No. 3, Group B, which is endorsed by the Department of Agriculture, Bureau of Chemistry. Therefore, a method which requires little time, is simple to perform, and at the same time just as specific must be given preference.

The outstanding test in Group B is No. 11. The relation of the specific gravity and the index of refraction is so markedly different in the two alcohols that it serves well for detecting methyl alcohol in ethyl alcohol. As a color reaction No. 9, Group B, is very good. Methyl alcohol on long boiling with hydroxylamine and alkali yields cyanide; ethyl alcohol does not. The cyanide produced is then easily detected by the usual reactions (p. 322).

All methods for the detection of methyl alcohol which have been proved to be due not to the methyl alcohol but to impurities found in the commercial product are omitted in this article. Among these may be mentioned (1) the property to prevent the precipitation of mercury by hydroxide (due to acetone), (2) the formation of iodoform in the cold with alkaline potassium iodide solution, (3) the production of a flocculent precipitate with Nessler reagent, and (4) the property to decolorize permanganate more rapidly.

After the above short discussion of the various methods for testing methyl alcohol, I will now indicate the procedure which experience has shown to be the best and the most reliable for detecting methyl alcohol in all kinds of liquors.

Typical Procedure for Methyl Alcohol Detection in Liquors.

Preparation.—100 cc. of liquor are taken in a 200 cc. distilling flask, neutralized with sodium carbonate solution to phenolphthalein, and slowly distilled until 50 cc. of distillate have been collected. The distillate is then divided into two portions, Distillate A, 30 cc., and Distillate B, 20 cc. The latter is tested directly as under No. 11, Group B, and No. 9, Group B.

Oxidation.—Add 100 cc. of 10 per cent sulfuric acid to Distillate A, using a 250 cc. distilling flask; also add 6 gm. of potassium dichromate and let stand for 10 minutes. Connect with a condenser and receiver and very slowly distill off 30 cc. The distillation of these 30 cc. should take 1 hour; the distillate then contains most of the acetaldehyde but very little of the formaldehyde, and is to be rejected. The distillation is then continued a little faster, until 60 cc. are obtained. This part of the distillate contains most of the formaldehyde and hardly any acetaldehyde. Some formaldehyde may still be left in the distilling flask, but the greatest portion is in these 60 cc. and this is used for the tests.

The tests in Group A can be subdivided into several groups, depending upon the reagents used. In the first group, phenylhydrazines are used as the reagent (Nos. 1 to 10); in the second, phenols (Nos. 11 to 23); in the third, alkaloids (Nos. 24 to 27); in the fourth, proteins (Nos. 28 to 34); in the fifth, amines (Nos. 35 to 39); and, lastly, there are a number of miscellaneous substances of varied structure (Nos. 40 to 46). It is my desire to select the most reliable tests and, if possible, choose them in such a way that each one is taken from a different subgroup. The advantage of such a set of reactions is obvious—each reagent used is of an entirely different structure; namely, hydrazine, phenol, protein, alkaloid, etc. It might be possible that a foreign substance in the suspected liquor will react somewhat like formaldehyde with one of the reagents, but it is not likely that the same substance will react in a manner similar to formaldehyde with all five of the reagents, each reagent being constructed differently. Furthermore, I never depend on color reactions alone; they should always be verified by tests which yield crystals, the latter being conclusive. Crystalline structure and melting point are definite and specific proof of the presence of a substance. I further amplify my proof by studying the physical properties of the alcohol, as described in No. 11, Group B.

Detection.—The tests are conducted in the following sequence.

1. No. 3, Group A; Color Reaction (1-4, 7, 8).—To 2 cc. of this oxidized fraction are added ten drops of a 5 per cent solution of phenylhydrazine hydrochloride, one drop of a 0.5 per cent sodium nitroprusside solution, and ten drops of 10 per cent sodium hydroxide solution. If formaldehyde is present, a blue color is obtained which changes to green and then to yellowish red. Acetaldehyde produces a red color.

2. No. 12, Group A; Color Reaction (19).—To 2 cc. of the material add two drops of 2 per cent phenol and carefully stratify sulfuric acid underneath. A red ring develops. Acetaldehyde gives a yellowish green, brown, or reddish brown ring.

3. No. 27, Group A; Color Reaction (53).—To 2 cc. of the oxidized material add 5 cc. of concentrated sulfuric acid, then add a few mg. of apomorphine. A reddish violet color develops.

4. No. 34, Group A, Color Reaction (64).—2 cc. of the oxidized material are heated to boiling after the addition of 0.08 gm. of peptone, three drops of 3 per cent ferric chloride solution, and its own volume of concentrated hydrochloric acid. If the original sample contained as little as 1 per cent of methyl alcohol, a bright violet coloration develops. Less peptone and less ferric chloride make it more sensitive.

5. No. 42, Group A; Color Reaction (79, 80).—To 2 cc. of the material add 1 cc. of concentrated sulfuric acid, cool, and add 5 cc. of fuchsin bisulfite solution; mix and allow to stand. The presence of formaldehyde is indicated by the violet-red color which gradually develops.

The fuchsin solution is made as follows.

Dissolve 0.5 gm. of fuchsin in 200 cc. of distilled water; add an aqueous solution of sulfur dioxide, the quantity corresponding to 1 gm. of sulfur dioxide gas; allow to stand until the solution assumes an amber color (this requires about 1 hour). The colorless fuchsin bisulfite solution which is obtained when more than 1 gm. of sulfur dioxide is added to 0.5 gm. of fuchsin is valueless as a reagent after standing 2 days. A solution prepared as outlined will keep well for 10 days, although it is recommended not to use a solution after 7 days.

6. No. 41, Group A; Crystals (Microscopic) (78).—10 cc. of the oxidized material are put into a small evaporating dish; 10 cc. of strong ammonia are then added. The mixture is evaporated, just to dryness, on the water bath. The formaldehyde condenses with the ammonia to hexamethylenetetramine. The latter is then dissolved in a few drops of water. A drop of mercuric chloride is added to a small amount of this solution and placed on a microscope slide. Typical crystals of hexamethylenetetramine-mercuric chloride develop. The shape of these cystals varies with the concentration. It is, therefore, best to make control experiments for comparison with the suspected material.

7. No. 11, Group A; Crystals and Melting Point (104).—Place in a test-tube 3 cc. of the oxidized distillate, 0.04 to 0.06 gm. of β-naphthol, and three to five drops of concentrated hydrochloric acid. Boil gently until the liquid becomes filled with an abundant precipitate of small white needles. Filter while hot. Wash with 1 cc. of dilute alcohol (1:2). Boil the precipitate with 4 cc. of dilute alcohol (1:1). (It is not necessary that all should dissolve.) Cool and filter off the precipitate. Wash with 1 cc. of dilute alcohol (1:1). Dry on porous tile in a warm place and determine the melting point.

Methylene-di-β-naphthol, the product, forms white needles which, when the temperature in the neighborhood of the melting point is raised at the rate of 1° in 15 seconds, begin to turn brown at 180°. It melts with decomposition to a red-brown liquid at 189–192° (uncorrected).

8. No. 11, Group B; Physical Properties (102).—Determine at 20°C. the refraction of the distillate obtained in the determination of alcohol by the immersion refractometer. If, on reference to the table, the refraction shows the percentage of alcohol agreeing with that obtained from the specific gravity, it may be safely assumed that no methyl alcohol is present. If, however, there is an appreciable amount of methyl alcohol, the low refractometer reading will at once indicate the fact. If the absence from the solution of refractive substances other than water and the alcohols is assured, this qualitative test, by difference in refraction, is conclusive.

The addition of methyl to ethyl alcohol decreases the refraction in direct proportion to the amount present; hence the quantitative calculation is readily made by interpolation in the table, using the figures for pure ethyl and methyl alcohol of the same alcoholic strength as the sample.

Example.—Suppose the distillate made up to the original volume of the measured portion taken for the alcohol determination has a specific gravity of 0.97350, corresponding to 18.38 per cent alcohol by weight, and has a refraction of 35.8 and 20°C. by the immersion refractometer; by interpolation in the refractometer table the readings of ethyl and methyl alcohol corresponding to 18.38 per cent alcohol are 47.2 and 25.4, respectively, the difference being 21.8; $47.2 - 35.8 = 11.4$; $(11.4 \div 21.8)\ 100 = 52.3$, showing that 52.3 per cent of the alcohol present is methyl alcohol.

9. No. 9, Group B; Color Reaction (100).—After specific gravity and refraction have been determined, take 20 cc. of the distillate and boil for 7 hours in a reflux apparatus with 15 gm. of potassium hydroxide and 1 gm. of hydroxylamine hydrochloride. This produces cyanide if methyl alcohol is present. The mixture is then cooled, acidified with sulfuric acid, and distilled with steam. The distillate is tested as follows.

To 5 cc. add a few drops of sodium hydroxide, a few drops of ferrous sulfate, and then ferric chloride. Finally, acidify with hydrochloric acid; if cyanide is present a Prussian blue color results. To a second 5 cc. portion add a few drops of sodium hydroxide, then add 2 or 3 cc. of yellow ammonium sulfide, and evaporate to dryness on the water bath. Cool and acidify with hydrochloric acid. Filter and add 1 cc. of ferric chloride. If a red color is produced, cyanide is present, indicating that methyl alcohol was originally present.

It might be well to mention the red hot copper spiral and odor test. It is extremely simple and is the only test which a traveling liquor inspector can perform. It depends on the production of formaldehyde when a red hot copper spiral is plunged into methyl alcohol. The odor, or, better, the sensation which formaldehyde produces in the upper part of the nose must then be recognized. For strong methyl alcohol whiskies this test is fairly good, but where it is a matter of 5 or 8 per cent of methyl mixed with ethyl alcohol or other substances found in liquors, it is extremely difficult, even impossible, for many people to detect the presence of methyl alcohol. I have tried this experiment on many individuals, using the same liquor, and found that some said methyl alcohol was present, others believed it to be absent, and still others were undecided.

Typical Procedure for Methyl Alcohol Detection in Tissues.

500 gm. of tissue are finely ground and put in a 1.5 liter distilling flask. 500 cc. of water are added. Enough sulfuric acid is added to make it distinctly acid. A few drops of mineral oil are then added to prevent frothing, and the mixture is distilled with steam. The distilling flask containing the material must be heated on a boiling water bath during this steam distillation. 300 cc. are collected. This distillate is then placed in a 500 cc. distilling flask neutralized to phenolphthalein, if necessary, and slowly redistilled, this time without steam. 100 cc. are collected and put into a 150 cc. distilling flask. 5 cc. of concentrated sulfuric acid are added and the mixture is then allowed to cool. 0.1 gm. of potassium dichromate is added and shaken to dissolve. Connect with condenser and receiver and slowly distill until 40 cc. are obtained. All the formaldehyde usually comes over in the first 40 cc., unlike that in the distillation of the oxidized liquors. The distillate is then used for the following reactions, 2 cc. being used for each of the first five tests, and 30 cc. for the sixth.

1. No. 42, Group A.—Fuchsin (reduced) plus sulfuric acid gradually develop a violet-red color. If no sulfuric acid is added, both acetaldehyde and formaldehyde give the reaction. For details, see page 321.

2. No. 3, Group A.—Phenylhydrazine hydrochloride plus sodium nitroprusside plus sodium hydroxide yield a deep blue color. For details, see page 320.

3. No. 24, Group A.—To 3 cc. of distillate add 5 cc. of concentrated sulfuric acid; mix; cool. Then add a few mg. of morphine. A violet-red color develops if methyl alcohol was originally present in the tissue.

4. No. 28, Group A.—To 3 cc. of distillate add 3 cc. of milk, then add a drop of very dilute ferric chloride and 5 cc. of concentrated hydrochloric acid. The mixture is then placed in a boiling water bath. A violet color develops if methyl alcohol was originally present in the tissue examined. Although this test is unreliable when examining liquors, it is quite trustworthy and characteristic when working with tissues. This is probably due to the organism having destroyed or conjugated and excreted the interfering substance originally present in liquors.

5. No. 23, Group A.—To 3 cc. of distillate add five or six drops of a saturated alcoholic solution of gallic acid and stratify some concentrated sulfuric acid underneath the mixture. A green to blue ring develops if methyl alcohol was originally present in the tissue.

6. No. 41, Group A.—Concentrated ammonia condenses with formaldehyde on evaporation into hexamethylenetetramine (urotropin). The latter gives characteristic crystals with mercuric chloride. For details, see page 321.

It is not necessary to obtain hexamethylenetetramine-mercuric chloride crystals to prove conclusively the presence of methyl alcohol in tissues. The reason for this is that the foreign substances found in liquors which cause false reactions are oxidized or conjugated and excreted rapidly from the organism, so that on distillation of the brain or liver tissue they are absent in the distillate.

SUMMARY.

1. The literature on methyl alcohol detection is reported.
2. 58 tests for the detection of methyl alcohol were critically studied to ascertain the reliability, specificity, sensitiveness, and influence of foreign substances upon them.
3. Over 250 liquors and over 700 human organs were analyzed for methyl alcohol.
4. The most reliable tests are pointed out.
5. A typical procedure for methyl alcohol detection in liquors is given in detail.
6. A typical procedure for methyl alcohol detection in tissue is given in detail.

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A METHOD FOR MANGANESE QUANTITATION IN BIOLOGICAL MATERIAL TOGETHER WITH DATA ON THE MANGANESE CONTENT OF HUMAN BLOOD AND TISSUES.

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In 1918, following an opportunity to examine a number of cases of chronic manganese poisoning occurring in a plant in the eastern part of the United States (1) it was decided to undertake chemical studies of the occurrence and distribution of manganese in normal subjects, in diseased subjects, and as opportunity permitted in cases of manganese poisoning. The first necessity of such a program lay in the development of a direct and simple method of manganese analysis. Such a method is now available and is reported in detail in the following paper. Its use has made possible the investigation of a variety of problems some of which are now under investigation in this laboratory and will be reported in subsequent papers, as for example the eventual distribution of insoluble non-toxic compounds introduced into the blood stream, and the points of excretion of metallic ions in the intestine.

Discussion of the Literature.

The literature on manganese readily discloses the fact that this element occurs in practically all living tissues. The literature from the time of Wurzer (2) contains occasional reports of manganese determinations in blood, many of which appear undoubtedly erroneous. However, in 1905, Bertrand began a series of exact scientific researches on the subject of manganese. These papers (3) deal with the effect of the catalytic action of traces of manganese on the growth of certain molds, plants, and bacteria. Bertrand concludes that managnese is of great physiological importance in plant and animal life.

In a paper on the normal manganese content of blood (4), Bertrand and Medigreceanu present an excellent review of the literature on this subject and report the following determinations of manganese expressed in milligrams per liter of blood.

	mg.
Man (2)	0.02
Sheep	0.06
Horse (3)	0.02
Steer (3)	0.02
Pig (3)	0.02
Rabbit (3)	0.02
Seal	0.02
Chicken	0.02
Duck	0.02

Determining the manganese separately in plasma and corpuscles in sheep blood, they find:

	mg.
Plasma	0.06 0.05
Corpuscles	0.025 0.02

In two later papers (5) they report several hundred analyses of various tissues of many invertebrates, fishes, and lower animals and invariably find manganese. The following table gives some of their results calculated on 100 gm. of fresh substance.

	Liver.	Kidney.	Lungs.	Eggs.
	mg.	*mg.*	*mg.*	*mg.*
Dog	0.306	0.106	0.010	
Ox	0.298	0.084	0.000	
Calf	0.290	0.063	0.011	
Pig	0.265	0.128	0.023	
Horse	0.289	0.077	0.006	
Rabbit	0.285	0.093	0.010	
Chicken	0.041	0.271	0.010	0.063
Duck	0.380	0.238	0.010	0.054
Frog	0.040			
Dogfish	0.089			

Bertrand points out the fact that the organs of principal functional importance, *e.g.* liver, kidney, etc., have the highest manganese content while the muscles, lungs, nerve tissues, etc. contain very little. He finds more manganese in the organs of birds than in mammals. There is some in the yolks of eggs and some in milk. From these data he concludes that manganese has an important physiological place in the living organism.

The method of analysis employed by Bertrand in these researches is briefly as follows (4, 6).

The tissue or blood is heated in platinum until nearly all the carbon disappears, the temperature being kept as low as possible to prevent the formation of the difficultly soluble manganese sesquioxide. The ash is treated first with a small amount of hydrochloric acid and warmed gently to dissolve any traces of sesquioxide which may have been formed, and then treated with sulfuric acid at a dull red heat. This treatment destroys the last trace of carbon and if not too strongly heated the manganese is left in easily soluble form.

Bertrand (6) discusses the various agents which have been proposed for the oxidation of manganese; *viz.*, lead peroxide by Crum (7), bismuth peroxide by Schneider,[1] potassium hypochlorite in the presence of copper sulfate by Duyk,[1] and alkaline persulfate in the presence of silver nitrate by Marshall (8). He finds the use of lead peroxide and the alkaline persulfates most satisfactory. In his hands the lead peroxide method, which consists of boiling a nitric acid solution of manganese with lead peroxide, allowing the dioxide to settle, and comparing the color formed with standards similarly prepared, gives results if exactly the proper experimental conditions are maintained; but he finds it very slow and troublesome and much prefers the alkaline persulfate method.

The alkaline persulfate method of Marshall (8) depends upon the following reaction, which takes place only when silver is present as a catalyst.

$$2Mn(NO_3)_2 + 5K_2S_2O_8 + 8H_2O = 2HMnO_4 + 10KHSO_4 + 4HNO_3$$

This reaction is due to the intermediate formation of silver peroxide, as proved by Bertrand (6). After considerable experimental work Bertrand varies slightly the conditions prescribed by Marshall and finally arrives at the following procedure. The residue from the sulfuric acid treatment is dissolved in nitric acid (sp. gr. 1.33) previously diluted with three volumes of water. The solution is made up to 10 cc. in a graduated tube, five drops of 10 per cent silver nitrate and 0.1 gm. of powdered potassium persulfate are added, and the whole is gently heated to boiling to develop the permanganate color and to decompose the excess of persulfate. This color is compared with standard solutions similarly prepared in standard comparison tubes. Bertrand states that only potassium persulfate can be used successfully and that ammonium persulfate always gives incorrect results (6).

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[1] Cited by Bertrand (6).

In 1907, Bradley proved manganese to be a normal constituent of the tissues and eggs of the fresh water clams, *Unio* and *Anodonta* (9). His analyses showed that the dry tissues contain about 1 per cent of manganese. Since in this case the manganese content was so high, he had no difficulty in applying a modified Volhard method. In his later paper (10) he develops a shorter titration method, wherein he oxidizes the manganese from the ash, with ammonium persulfate in a nitric acid solution in the presence of silver as a catalyst, to permanganic acid. After boiling, he titrates the permanganic acid with a standard solution of arsenious acid. Although specimens of these clams obtained from all parts of the United States were analyzed not one was found that was manganese-free, nor did the amount present vary greatly. Bradley also showed that in water where the manganese content was very low the clams were of smaller size than in those waters where the manganese was more plentiful, thus indicating that manganese was a necessary element in their metabolism.

Method.

Since it has been shown that the blood and tissues contain only a very small amount of manganese, and, since the limit of sensitivity of the colorimetric method is 0.002 mg., it is evident that if results of value are to be obtained the sample of material taken for analysis must be fairly large, 20 to 100 gm. The destruction of this large amount of organic material without loss of the small amount of manganese becomes a matter of some difficulty.

There are two principal operations to be considered in the discussion of any process for the determination of small amounts of manganese in blood and animal tissues; (1) the ashing and subsequent solution of the material, and (2) the regulation of conditions for the oxidation of the manganous salts to permanganic acid. These two operations will be discussed separately and in the above order.

Ashing and Subsequent Solution of Material.

When we first tried Bertrand's method we feared low results on account of the difficulty of dissolving the ignited ferric oxide of the ash in hydrochloric and sulfuric acids at a low temperature and felt it would be safer to take up the ash with an acid fusion, which is essentially treatment with sulfuric acid at 500–600°C.

The first problem then was the choosing of a receptacle suitable for this fusion. In the literature the ashing process is nearly always described as being carried out in platinum at as low a temperature as possible. In such a slow process the expense is prohibitive if many analyses are to be made at a time. There is also a possibility of solution of some of the platinum, which is likely to interfere in the final color determination. For these reasons we have not considered platinum, and there remained a choice of porcelain, Pyrex glass, or quartz. We have tried all three of these.

Small porcelain dishes of a variety of makes gave good results except that breakage was frequent and the inside glaze had a tendency to chip after being used a few times.

With Pyrex glass we had rather high breakage and the glass would often soften when the dry ashing and fusion process was used. However, better results were obtained with what we may call the wet process; *i.e.*, destruction of the organic material with mixtures of concentrated sulfuric, hydrochloric, and nitric acids in a Pyrex Kjeldahl flask. This process would admit of no question as to complete recovery of manganese from the ash, neither was there any danger of contamination, as repeated tests have shown that Pyrex glass is free from manganese. The tissue or blood was boiled a short time with concentrated sulfuric acid to remove water and fat and then 30 to 40 cc. of concentrated nitric and hydrochloric acids were added and the mixture was allowed to stand over night. In the morning, it was usually necessary only to boil off the excess acid to obtain a few cc. of clear liquid containing all the manganese in solution as sulfate. A large number of determinations and recoveries on blood and tissues have been made by this method with uniform success and while we feel that it is absolutely reliable there are obviously several serious drawbacks; *i.e.*, the length of time, the large amount of reagents required, and the difficulty of fume disposal when large numbers of analyses are to be made.

Therefore we decided to try the remaining possibility; *viz.*, quartz beakers. The only difficulty with the quartz beakers now upon the market is that many contain manganese and as the acid sulfate fusion dissolves some of the beaker contamination results. This manganese probably enters the beaker as an impurity in the raw material. We have discussed this matter with the manufacturers and are assured that beakers free from manganese will soon be available. In the meantime, a number of beakers are purchased, blank fusions made in them all, and those containing manganese are returned. In a dozen beakers, there are usually one or two which show a trace of manganese, about 0.002 mg; while occasionally one will give a blank of ten times that amount. Because each fusion removes a thin layer of quartz and because there is no certainty that the manganese content is *nil* throughout the thickness of the beaker, several blanks must be made in the same beaker during the period of its use, but so far such blank fusions have repeated the initial result.

The following is the exact procedure in what may be called the quartz beaker method. In the case of blood, the sample for analysis is taken directly from a vein by means of an all glass syringe and placed in a tared quartz beaker. In the case of tissues, they are carefully washed and cleaned of adhering portions of fat and connective tissue, rinsed finally with distilled water, put into a tared beaker, and weighed. Wet weights are used throughout. In case the blood is obtained outside the laboratory, we supply clean bottles containing a small amount of pure potassium oxalate to prevent coagulation of the blood. From this point, blood and tissues are treated alike. The beaker containing the wet material is heated on a sand bath until it is thoroughly dried.

Then it is put over a Tirrill burner so that the hottest part of the flame strikes the mouth of the beaker, not the bottom, as too high a temperature will fuse the salts in the blood and tissue into a glass which is very difficultly soluble. The beakers are frequently turned and heated in this manner until the tar which forms has completely dried out. Then they are put into an electric furnace maintained at 600–700°C. until the carbon has nearly disappeared. This point should not be overstepped because of the possibility of fusing the ash into a very resistant glass. In fact, a considerable amount of carbon left at this point does no harm as it will be filtered out later on. It is graphitic carbon and cannot interfere with subsequent work.

The ash is now ready for the acid sulfate fusion. Ordinarily, fused potassium acid sulfate would be used ($K_2S_2O_7$). It is difficult, however, to obtain this compound manganese-free, since it is usually fused in iron containers which invariably contain manganese. We have made the acid sulfate directly in the beaker at the time of fusing by treating 2 to 4 gm. of a mixture of sodium and potassium nitrate with 10 cc. of pure sulfuric and 5 cc. of hydrochloric acids and warming gently. The mixture of nitrates is used in order to obtain a lower melting point. This procedure has several advantages. The nitrates and acids are readily obtained pure. The nitric acid set free and the hydrochloric acid destroy much of the residual carbon and are better solvents for some of the ignited salts than is sulfuric acid alone. The excess acid wets the material evenly and, while boiling, some of it condenses on the sides of the beaker and carries down adhering material. Very soon a few cc. of a clear fusion form at a cherry-red heat. Most of the carbon left from the ashing is destroyed before this point is reached. However, if a large quantity is still present it is well to add a few cc. of hydrochloric or nitric acid or both, and a few drops of sulfuric acid, and again heat until the whole fusion is red. This will attack any mineral matter within the carbon particles.

When the fusion is finished it should be liquid with a few crystalline flakes on the surface. The beakers should be rotated with tongs so that the molten material will flow round the sides and not harden in a solid mass at the bottom, for potassium sulfate expands sharply as it solidifies and the beaker is sure to crack. This is very important. If too much sulfuric acid is boiled off and the melt hardens at the high temperature of the Tirrill burner, it must be removed from the flame and cooled for about 2 minutes and then a few cc. of sulfuric acid cautiously poured down the side of the beaker. The acid is then evaporated as before.

After the melt has hardened and nearly cooled, 50 cc. of water are added and the beaker is set on a sand bath until the contents have dissolved. If the cold water is poured onto a thick layer of the solidifying sulfates while they are still very hot, the beaker is likely to crack locally in the vicinity of the lump. Often there are white flocculent particles of silica and black specks of carbon floating in the solution at this point. These should be filtered off and the liquid received in a Pyrex conical flask. The silica is present in large quantities if the fusion has been carried too far, because some of the normal sulfates lose sulfur trioxide leaving an alkali behind which attacks the silica of the beaker at the high temperature of the fusion. It is when this happens that a beaker containing manganese would seriously contaminate the analysis. Sometimes this silica passes through the filter and eventually makes the final color comparison difficult, the solution having a greenish color due to colloidal silicic acid. If 1 cc. of hydrofluoric acid which has been previously tested for manganese is added and the solution warmed, this silica will immediately disappear. If the volume is greater than 50 cc., the solution is evaporated on a sand bath to approximately this volume, and is then ready for the final step, the development of the permanganate color.[2]

The Oxidation of Manganese Salts to Permanganic Acid.

Like Bertrand, we found that both the lead dioxide and persulfate methods of oxidation gave correct results and we also had very good success with the bismuthate method used in steel analysis. We used this last method for a time but as it involves filtration by suction in each determination it was time-consuming and the shorter persulfate method was employed giving results which checked perfectly with those obtained by the longer bismuthate method.

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[2] It is sometimes desirable to clean the beakers thoroughly after a series of determinations to remove the black film of carbon which often forms and any bits of silica which may have been loosened by the previous fusions. We have found that the easiest way of doing this is to put about 10 cc. of hydrochloric and 5 cc. of hydrofluoric acids into the beaker and gently warm while rotating with tongs to bring the acid in contact with all parts of the surface. A dozen or more beakers can be thoroughly cleaned in a very short time with the same acid. If this washing is done rapidly the amount of silica which is removed is very small.

Bertrand (6) holds that potassium persulfate is far superior to the ammonium salt and that the nitric acid concentration should be kept very high (one part of concentrated nitric acid to three parts of water). We have used both potassium and ammonium persulfate with equal success except in the instance of two bottles of the ammonium salt which, although of best quality, contained a large amount of organic matter. This impurity, of course, reduced the permanganate to insoluble manganese dioxide. A wide range of acid and silver nitrate concentrations have been tested and consistent success has been obtained with the one recommended below, and fair success together with unexplained failures in using stronger acid solutions.

We recommend 1 cc. of concentrated nitric acid and 0.2 to 0.4 cc. of 2.5 per cent silver nitrate for 50 cc. of solution. For ease in pipetting, the acid is diluted to 50 per cent (by volume) and 2 cc. are used. The acid and silver nitrate are added to the solution containing manganese together with about 0.5 gm. of persulfate and the whole is warmed gently on the sand bath for 10 minutes. Bertrand recommends boiling at this point in order to destroy the excess persulfate which he believes often oxidizes the silver to silver peroxide. We do not consider this important, and boil our solution only a short time if at all, and find that the permanganate color will keep unchanged for several days if a small excess of persulfate is allowed to remain. With turbid solutions which are difficult to compare, this is an advantage for the solution may be allowed to stand over night, and then the clear pink solution can easily be decanted into a Nessler tube leaving the residue behind. If it is desirable to determine the amount of color in a turbid solution without delay, the liquid may be centrifuged for 2 to 3 minutes, and the clear supernatant liquid decanted into a Nessler tube.

The standards used for comparison will keep unchanged for several weeks if the color is developed as described above, and if the tubes are kept covered to protect them from the dust. Standards made up by diluting a potassium permanganate solution of known concentration will not keep, however carefully the water used for diluting has been prepared. As potassium permanganate is the salt of manganese most easily obtained pure it is well to start with a calculated and weighed amount of it and reduce the manganese to the manganous state by adding a solution of sulfurous acid. The whole is made up to a known volume. Fractional parts of this stock solution are taken from time to time, 2 cc. of the 50 per cent nitric acid, a few drops of 2.5 per cent silver nitrate, and 0.5 gm. of persulfate are added, the volume is made up to 50 cc., and the color is developed by 10 minutes heating. In this manner Nessler tubes containing 0.002, 0.004, 0.006, 0.008, 0.010, 0.012, 0.015, 0.020, and 0.025 mg. of manganese are prepared.

If there are more than 2 or 3 mg. of manganese in the sample for analysis, manganese is likely to be precipitated in the form of manganese dioxide unless a different set of conditions is maintained; *i.e.*, stronger acid—more silver and persulfate. If this brown precipitate is not too great in amount it may usually be dissolved and converted to the permanganic acid by adding 1 or 2 gm. of the persulfate. This, of course, increases the acidity and dissolves the manganese dioxide immediately, oxidizing it to permanganic acid. If the precipitate is heavy it is advisable to dissolve it by the addition of a small amount of sulfurous acid and develop the color in an aliquot part.

When the color in the sample runs higher than the highest tube, we generally dilute to 100 or 200 cc. with distilled water and take 50 cc. for comparison. A Duboscq colorimeter has been employed for stronger solutions using suitable standards which are prepared 500 cc. at a time and kept in the dark in glass-stoppered bottles. These stronger solutions (0.2 and 0.3 mg. of manganese per 100 cc.) have kept for several weeks without change.

It is, of course, almost unnecessary to note that chlorides or other reducing agents must be absent from the solution in which the permanganate color is developed, or to say that all reagents must be proved to be absolutely free from even traces of manganese before they can be used in this work.

Using the method described above, a series of experiments employing a constant amount of ox blood (50 gm.) to which varying amounts of manganese had been added has been made and has given uniformly good results. The results of this series are given in Table I. In the course of our work we have made a large number of other recoveries and checks which are not reported. At the same time we performed a parallel series of experiments using Bertrand's method of alternate treatment with hydrochloric and sulfuric acids in order to prove whether or not our suspicions in regard to the interference of iron in that procedure were well founded. A comparison of the results by the two methods shows that, while recoveries are possible by Bertrand's method, more consistently good recoveries are made by the shorter and simpler acid fusion method.

TABLE I.

Acid fusion method.		Bertrand's method.	
Mn added.	Mn recovered.	Mn added.	Mn recovered.
mg.	*mg.*	*mg.*	*mg.*
0.003	0.004	0.003	0.003
0.003	0.003	0.003	0.000
0.003	0.002	0.003	0.003
0.004	0.005	0.004	0.000
0.005	0.005	0.005	0.002
0.005	0.006	0.005	0.002
0.005	0.006	0.005	0.005
0.005	0.005	0.005	0.006
0.005	0.005	0.005	0.003
0.005	0.006	0.008	0.002
0.005	0.004	0.008	0.007
0.008	0.009	0.010	0.006
0.010	0.011	0.010	0.007
0.010	0.012	0.010	0.006
0.010	0.010	0.013	0.008
0.013	0.013	0.020	0.012
0.013	0.013	0.020	0.013
0.020	0.023		
0.020	0.021		
0.020	0.019		
0.020	0.020		

TABLE II.

Manganese Content of Human Blood.

Name.	Weight of sample.	Mn per 100 gm.	Remarks.	Name.	Weight of sample.	Mn per 100 gm.	Remarks.
	gm.	*mg.*			*gm.*	*mg.*	
R. B. C.	42.5	0.010		C. K. D.	27.1	0.013	Nov., 1919.
J. W. M.	20.0	0.015			25.0	0.010	" "
S. G.	20.0	0.010			27.0	0.012	Dec., "
G. E. D.	20.0	0.015			27.3	0.014	" "
H. L. R.	20.0	0.010			28.0	0.012	Jan., 1920.
T. P. K.	20.0	0.005		W. H.	37.5	0.008	" "
L. M. D.	20.0	0.010			33.8	0.009	" "
F. C. F.	20.0	0.010			37.5	0.008	" "
L. E. W.	20.0	0.005		J. Q.	25.1	0.019	Early syphilis.
E. J. R.	24.0	0.017		W. C.	22.4	0.018	" "
R. E. L.	25.0	0.020		D. W.	22.4	0.013	" "
H. I. B.	20.0	0.015		E. S.	23.1	0.017	" "
G. W. T.	22.0	0.004		E. A. R.	14.4	0.021	" "
M. L. S.	26.8	0.013		G. K.	23.9	0.020	Sprue.
W. O. F.	25.1	0.010	Oct., 1919	S. T.	9.3	0.015	Nephritis.
	25.5	0.011	Dec., "	G. A.	9.8	0.020	"
	29.5	0.010	" "	A. J.	26.2	0.023	Anemia.
	26.5	0.011	" "	M. A. P.	16.0	0.025	"
	29.2	0.010	" "	M. C.	22.6	0.013	" Jan. 5, 1920.
	21.8	0.013	" "		33.0	0.012	Jan. 5, 1920.
A. S. M.	100.0	0.013	Oct. 6, 1919		30.8	0.018	" 15, "
	27.7	0.011	" 27, "	T. M.	25.7	0.010	Pernicious anemia.
	28.9	0.010	Dec., 1919	M. C.	30.5	0.010	" "

A. S. M.	29.0	0.014	Dec., 1919	Min.	16.0	0.025	Acute leucemia before transfusion.
	29.1	0.010	" "		9.3	0.020	" " after "
	34.0	0.014	Jan., 1920	H. M. K.	31.6	0.006	Syphilis.
	32.2	0.012	" "	Pric.	25.7	0.008	"
C. K. R.	25.0	0.012	June, 1919	M. M.	21.1	0.010	Secondary syphilis.
	28.2	0.010	Dec., "	F. P.	22.8	0.004	" "
	37.2	0.010	Jan., 1920	W. S.	24.3	0.004	Syphilis of central nervous system for 15 yrs.
	19.3	0.010	" "		23.5	0.006	
L. F.	25.0	0.012	June, 1919	H. W.	23.2	0.009	Syphilitic aortitis.
	34.0	0.009	Jan., 1920	S. W.	20.8	0.009	Secondary syphilis.
R. M. T.	26.0	0.010	Dec., 1919	P. R.	22.0	0.005	Syphilis 15 to 20 yrs.
	26.7	0.011	" "	M. A.	22.1	0.009	Cirrhosis of liver (syphilitic).
	28.6	0.011	" "				
	30.0	0.010	" "				
L. A. S.	24.0	0.010	" "				
	29.7	0.008	" "				
	26.0	0.012	" "				

Normal Manganese Content of Human Blood.

The manganese in the blood of a number of persons not exposed in any way to manganese has been determined and the results are given in Table II. The results with no legend attached are from blood specimens obtained from members of the staff and student body at the Medical School. The others are from hospital patients in various pathological conditions.

In those cases where several analyses were made on blood obtained at different times from the same individual the results serve to show the reliability of the method and the general constant level of manganese in the blood.

Although the number of analyses from cases of anemia is not large enough to warrant final conclusions, no marked decrease in manganese content is indicated, as has been claimed by earlier investigators (11). In fact no abnormality in manganese content is shown by our few pathological figures except perhaps in the cases of syphilis of long duration where the manganese is low.

Our results are from five to ten times as high as those reported by Bertrand for human blood. This discrepancy we believe to be due to the difficulty of recovering all the manganese from the ash of blood by Bertrand's method as we have pointed out, or possibly to the difficulty of making the color comparison in such a small volume (10 cc.) with the large amount of iron present. The only other explanation is that the manganese content of the blood of Bertrand's subjects was actually lower than that of the blood we analyzed. In some of our experimental work to be published later we have shown that the amount of manganese in the blood can be increased for a short time by the ingestion of manganese-containing ores. If the normal food consumed here contains more easily soluble manganese than that eaten by Bertrand's subjects there is a possibility that the blood content of manganese might vary in the two countries. The surface waters of eastern Massachusetts contain from 0.001 to 0.015 parts of manganese per 100,000.[3] This might be taken as a rough indication of the amount of manganese consumed here. Unfortunately we have no such data from France with which to make a comparison.

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[3] Personal communication from Dr. H. W. Clark, Director and Chemist of the Division of Water and Sewage Laboratories, Massachusetts State Department of Health.

In Table III are given the results of the manganese content determinations in human tissue from a limited number of autopsies. None of these individuals had been in any way exposed to abnormal quantities of manganese, and therefore the results may

TABLE III.

Manganese Content of Human Tissue.

Weights of samples are for wet material.

Tissue.	No. 1, adult.		No. 2, adult.		No. 3, baby.		No. 4, adult.		No. 5, baby.	
	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.
	gm.	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*
Stomach.......			35.0	0.020	31.1	0.013				
Small intestine.			75.0	0.027	14.4	0.017			7.5	0.053
Colon..........			60.0	0.050	17.3	0.022			10.7	0.018
Liver..........	80.0	0.125	63.0	0.160			33.2	0.105	11.4	0.154
Kidney........	53.0	0.070	53.0	0.060	8.0	0.052	17.2	0.046	15.3	0.052
Spleen.........	46.0	0.027	56.0	0.030	3.6	0.053				
Pancreas.......										
Adrenals.......			21.0	0.013						
Lymph nodes..										
Lung..........					14.3	0.019	25.4	0.047	14.2	0.014
Muscle.........										
Brain..........					17.7	0.029				
	No. 6, baby.		No. 7, adult.		No. 8, baby.		No. 9, adult.		No. 10, child (4 yrs.).	
Stomach.......			26.9	0.030	19.3	0.020	50.8	0.040	41.3	0.027
Small intestine.	39.4	0.057	42.0	0.021			67.1	0.017	24.6	0.023
Colon..........			41.2	0.065	34.5	0.065	68.8	0.011	33.1	0.010
Liver..........	46.6	0.368	39.9	0.263	19.0	0.185	59.5	0.137	69.3	0.123
Kidney........	24.3	0.094	33.0	0.052	6.1	0.049			36.5	0.068
Spleen.........	5.5	0.055	46.0	0.026			33.8	0.051	30.6	0.026
Pancreas.......							24.1	0.100	13.3	0.075
Adrenals.......										
Lymph nodes (peribronchial)........							8.0	0.063		
Lung...........	22.5	0.027	42.3	0.045	11.2	Trace.	38.8	0.010	50.3	0.010
Muscle.........							20.4	0.014		
Brain..........									72.1	0.024

TABLE III—*Concluded.*

Tissue.	No. 11, adult.		No. 12, anemic adult.		No. 13, tubercular adult.		No. 14, adult.	
	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.
	gm.	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*
Stomach	35.1	0.028	24.9	0.028				
Small intestine	33.1	0.027	30.8	0.013	19.0	0.036	12.0	0.025
Colon	64.9	0.046	31.7	0.035	36.1	0.022	21.0	0.019
Liver	50.7	0.168	79.3	0.101	64.5	0.217	54.0	0.111
Kidney	3.3	0.120	44.4	0.047	70.9	0.031	58.0	0.052
Spleen	23.5	0.029	50.8	0.016			27.0	0.022
Pancreas	17.1	0.093	38.6	0.070	28.8	0.055	23.5	0.060
Adrenals								
Lymph nodes								
Lung	46.9	0.019			21.1	0.024	48.0	0.004
Muscle								
Brain			68.5	0.032				
Heart							50.4	0.021

Tissue.	Mean of the above results.	No. of figures taken in mean.
	mg.	
Stomach	0.026	8
Small intestine	0.029	11
Colon	0.033	11
Liver	0.170	13
Kidney	0.061	13
Spleen	0.032	10
Pancreas	0.076	6
Adrenals	0.013	1
Lymph nodes	0.063	1
Lung	0.020	11
Muscle	0.014	1
Brain	0.028	3
Heart	0.021	1

properly be classed as normal. It will be noted that the results for any one tissue vary considerably. We consider this to be quite normal as the same variation is found in the tissues of other animals, and is probably dependent on the food and environment of the individual. The average of all the results on each tissue is given at the end of the table. The number of analyses is too small to permit of finding a true average but the figure is at least close to the correct value.

CONCLUSIONS.

1. A method is developed for the analysis of manganese in blood and tissue which is more rapid and has fewer sources of error than methods heretofore employed.

2. A series of results is given for the manganese content of human blood. All these are from normal subjects as far as exposure to manganese is concerned, although some of the individuals from whom blood samples were taken were pathological cases. A number of check results on the same individuals taken at different times show the general constant level of manganese in the blood.

3. A series of results for the manganese content of human tissue obtained from fourteen autopsies is presented. Manganese was found in all the tissue analyses, the liver carrying the highest amount, averaging 0.170 mg. per 100 gm. of wet tissue.

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BLOOD SUGAR CONCENTRATION AND BLOOD SUGAR METHODS.

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By blood sugar concentration is meant the content of glucose in the blood. Our knowledge of the amount of glucose in the blood is derived almost exclusively from reduction methods. Besides glucose, however, the blood contains other reducing substances; *viz.*, fructose, ketones, uric acid, creatinine, etc.

Whether the blood sugar values obtained by the reduction methods give only the amount of glucose or whether other reducing substances also play a part in the reduction has often been discussed.

Determination of the actual amount of glucose in the blood is not easy. One cannot rely on polarimetry, as the blood contains several substances which act optically. It is best to ferment the blood and determine the reduction before and after. But the fermentation also may introduce errors. In part the yeast may contain reducing substances, or these may be formed during fermentation; in part the fermentation of glucose is, as a rule, more or less incomplete; and in part it may well be supposed that the reducing substances of the blood which are not subject to fermentation may be converted into non-reducing substances when the blood is in process of fermentation.

Several investigators have attempted to ascertain whether fermented blood contains a reducing substance, and whether a so called residual reduction exists after fermentation. They have obtained discordant results, which seem to be due partly to the methods used. Thus Frank and Bretschneider (1) found no residual reduction by Bertrand's method, either in normal people or in diabetics with hyperglycemia. Maase and Tachau (2) found substantial agreement between Bertrand's copper method, Tachau's mercury method, and polarization, both in normal subjects and in diabetics.

On the other hand, Schumm (3) found an inconsiderable residual reduction by Bang's old method, which, however, Bang himself admitted gave too high values. Further, Ege (4) asserts in a recently published work that both Bang's micro method, in the modification employed by Ege, and Hagedorn and Jensen's method (5) give residual reduction, although with both methods this is so slight that in normal persons it has no practical, clinical importance. In the uremic, however, the residual reduction may be great, but, as the analyses have not been published, it is impossible to tell how great.

Stepp (6) has followed another course in order to decide whether the blood sugar values found by the reduction methods really are due only to glucose. If the protein and other colloids of the blood are precipitated with phosphotungstic acid, and the carbon in the blood is determined by elementary analysis, one finds the so called residual carbon, which in normal individuals amounts to 150 to 200 mg. per 100 cc. of blood. Since glucose contains 40 per cent of carbon, about 40 mg. of this residual carbon comes from glucose, if one assumes that the amount of glucose in the normal individual is about 0.1 per cent. If the blood sugar concentration increases in diabetics to several times the normal, one would expect that whenever the quantity of glucose is increased by 0.1 per cent the residual carbon must increase by 40 mg.

In a series of diabetics with hyperglycemia Stepp has determined the bood sugar concentration by Bertrand's method, while he has determined the residual carbon by elementary analysis. He made the following observations.

In a number of diabetics the residual carbon increased parallel with the blood sugar, so that the increase of the blood sugar found was probably due to glucose. In others the blood sugar values rose much more than the residual carbon, so that the blood sugar values found were certainly too high. In a third group a higher amount of carbon was found than one would expect according to the reduction. This was found in patients with acidosis and was probably due to increase in the ketone bodies of the blood.

From these investigations it appears that in diabetic hyperglycemia other substances besides glucose may form, which reduce copper by Bertrand's method, so this method also may give too high values.

During the last 5 or 6 years a number of new methods have been described which seek to determine in a simple manner the glucose concentration in a minimum of blood.

From Danish sources two comparative investigations of newer blood sugar methods have already been published; Leshly (7) finding that Bang's and Benedict's methods showed results which agreed, and Ege (4) finding that Bang's method as well as Hagedorn and Jensen's method gave approximately the same values, though the results obtained by the former method were somewhat lower than by the latter.

A few years ago one of us (Höst) began a number of comparative investigations, which were not completed, by Benedict's method and Bang's method, and found that Benedict's method in many cases of diabetes gave higher values than Bang's method. These results therefore did not agree with those of Leshly.

As Folin and Wu (8) also have recently published a colorimetric micro method, and Bang's method in its latest modification, as far as we know, has not been compared with other methods, we decided to undertake comparative investigations of blood sugar values in normal and sick individuals, especially diabetics, by the following four methods:

1. Bang's method, in its latest modification, worked out by Bang and Hatlehoel (9).
2. Hagedorn and Jensen's (5) method, which is referred to as Hagedorn's method.

Hagedorn and Jensen's blood sugar method has been published only in Danish, and we therefore give a résumé of it here.

Reagents.

1. Potassium Ferricyanide Solution.—1.649 gm. of potassium ferricyanide and 28.6 gm. of crystalline sodium carbonate are dissolved in 1,000 cc. of water. In a black bottle this solution will keep for 2 months.

2. Potassium Iodide Solution.—5 gm. of potassium iodide free from iodate, 10 gm. of zinc sulfate, and 50 gm. of sodium chloride are dissolved in 200 cc. of water.

3. Acetic Acid.—3 per cent acetic acid.

4. Starch.—1 gm. of starch dissolved in 100 cc. of water with thorough boiling.

5. Sodium Thiosulfate Solution.—1.24 gm. of sodium thiosulfate dissolved in 1,000 cc. of boiled water.

6. Protein Precipitate.—0.1 N NaOH and 0.45 per cent zinc sulfate (0.45 gm. of crystalline zinc sulfate dissolved in 100 cc. of water).

For the test 0.1 cc. of blood is pipetted (capillary pipette) into a test-tube containing 1 cc. of 0.1 N NaOH and 5 cc. of zinc solution. The pipette is rinsed with the zinc-NaOH mixture. The test-tube is then placed in boiling water for 4 minutes, cooled, and the contents are filtered through cotton into a test-tube or a small flask. The residue is washed twice with 3 cc. of water. From a micro-burette are added exactly 2 cc. of the potassium ferricyanide solution. The test-tube or flask is then placed in boiling water for 15 minutes, cooled, and 3 cc. of potassium iodide solution and 2 cc. of acetic acid are added. From a micro-burette the thiosulfate solution is added till the yellow color disappears and then, after adding one drop of starch solution, till the red-blue color just vanishes.

Calculation.

The volume in cc. of thiosulfate solution used multiplied by its factor is subtracted from 2.00. The blood sugar in per cent, corresponding to the used amount of potassium ferricyanide, is found in the table. The value found in a blank analysis is subtracted from the blood sugar found.

Table for Hagedorn and Jensen's Blood Sugar Method.

Used potassium ferricyanide solution.	Glucose in 100 cc. of blood.
cc.	*mg.*
0.10	0.017
0.20	0.035
0.30	0.053
0.40	0.070
0.50	0.088
0.60	0.106
0.70	0.124
0.80	0.141
0.90	0.159
1.00	0.177
1.10	0.195
1.20	0.214
1.30	0.232
1.40	0.251
1.50	0.270
1.60	0.290
1.70	0.310
1.80	0.331
1.90	0.335
2.00	0.384

3. Myers and Bailey's (10) modification of Lewis and Benedict's method, referred to as Benedict's method.

4. Folin and Wu's (8) method, referred to as Folin's method.

By these four methods we have determined the sugar concentration in thirty-five samples of blood from twenty-eight normal persons and patients.

We have divided the analyses, Hatlehol using Bang's and Folin's methods, while Höst employed Hagedorn's and Benedict's methods.

The blood was obtained by venous puncture, and was kept fluid by a little pulverized potassium oxalate.

The individuals examined may be divided into three groups: (1) non-diabetics, such as convalescents and patients suffering from slight affections, which would not influence the blood sugar (Table I); (2) non-diabetics, with affections which might possibly cause changes in the blood sugar (Table I); and (3) diabetics (Table II).

The values noted in the analyses are expressed in gm. per 100 cc. of blood. Since in Bang's and Hatlehol's methods, as is known, the blood is weighed, the values are reckoned by this method to mean 100 cc. of blood by adding 6 per cent to the value found.

As will appear from the analyses, we have as a rule undertaken double determinations, since in addition to a determination directly in the oxalated blood we have also determined the sugar concentration in a measured amount of blood with a certain quantity of glucose added. We compared the found and calculated sugar concentrations in the blood samples which had an addition of glucose. The results are given in Table III.

The accuracy of the colorimetric methods is of course dependent on the colorimeter. As we used a Bock-Benedict colorimeter which was not especially accurate, the limits of error found for the colorimetric methods were undoubtedly higher than they would have been if we had used a more accurate instrument.

In all four methods the quantities of glucose added, which varied from 0.03 to 0.16 per cent, are, within the above stated error limits, recovered.

As appears from the analyses, different blood sugar concentrations are found by the four methods. The differences may to some extent be due to the inaccuracy of the methods. But even if one reckons with the greatest error found, 10 per cent, this is far from sufficient to explain the difference between the results of the various methods, since this is in most cases considerably greater.

As will be seen there is a certain regularity in the different results given by the methods. In the majority of cases the colorimetric methods give higher values than the titrimetric, and as a rule the values are larger for Benedict's method than for Folin's, which agrees with the statement of Folin and Wu (8). The titrimetric methods, Bang's and Hagedorn's, give approximately identical values. The difference between the results from these two methods is in most cases within 10 per cent.

The difference in the blood sugar concentration found by the four methods is considerably more pronounced in diabetics than in normal persons and non-diabetics. That these differences are not due to accidental circumstances is shown best by Nos. 34, 35, and 36, which are taken from the same patient, but on different days. In these three determinations very different results were found by the four methods, but each time the greatest values were found by Benedict's method, somewhat smaller by Folin's method, still smaller by Hagedorn's method, and smallest by Bang's method.

As the glucose added is recovered in the blood by all the methods employed, the difference, in some cases considerable, which is found by the use of the different methods, cannot be due to a loss by the methods which give the smallest results. The cause must be that substances other than glucose also effect reduction by the methods which give the highest values; that is, the colorimetric methods. How far the two titrimetric methods, Bang's and Hagedorn's, give only the amount of glucose, or whether other reducing substances also play a part in the result by these methods does not appear from the investigations.

We will not enter into the question as to what other substances besides glucose effect reduction by the colorimetric methods. We shall only mention that the diabetic whose blood showed the greatest differences in the blood sugar, as determined by these methods (*cf.* Nos. 34, 35, and 36), had no acidosis, and the same of course is true of No. 13, who had only a little bronchitis, and in whose blood Benedict's method gave considerably higher values than the other methods.

TABLE I.

Non-Diabetic Subjects.

Subject.	Method.	Blood as drawn.	Blood + known amounts of glucose.		
			Addition.	Found.	Calculated.
		per cent		*per cent*	*per cent*
1. L. A., 30 yrs., dyspepsia.	Bang.	0.070			
	Hagedorn.				
	Benedict.	0.075			
	Folin.	0.085			
2. H. A., 25 yrs., palpitation.	Bang.	0.100	10 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.189	0.182
	Hagedorn.	0.100		0.175	0.182
	Benedict.	0.102		0.190	0.184
	Folin.	0.095		0.182	0.177
3. V. J., 52 yrs., vitium cordis.	Bang.	0.097			
	Hagedorn.	0.091			
	Benedict.	0.118			
	Folin.	0.105			
4. B. S., 45 yrs., pyuria.	Bang.	0.084	10 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.164	0.167
	Hagedorn.	0.094		0.179	0.185
	Benedict.	0.090		0.185	0.181
	Folin.	0.102		0.190	0.184
5. H. N., 28 yrs., convalescent.	Bang.	0.084	7 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.197	0.198
	Hagedorn.	0.077		0.204	0.192
	Benedict.	0.098		0.217	0.211
	Folin.	0.089		0.204	0.203
6. K. F., 50 yrs., dyspepsia.	Bang.	0.099	6 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.186	0.199
	Hagedorn.	0.105		0.198	0.204
	Benedict.	Lost.		0.217	
	Folin.	0.108		0.216	0.206
7. R. J., 23 yrs., hematuria.	Bang.	0.091	6 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.189	0.193
	Hagedorn.	0.097		0.181	0.198
	Benedict.	0.118		0.225	0.214
	Folin.	0.105		0.214	0.204
8. M. S., 54 yrs., paraparesis.	Bang.	0.099	10 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.156	0.166
	Hagedorn.	Lost.		0.152	
	Benedict.	0.093		0.174	0.161
	Folin.	0.099		0.157	0.166

TABLE I—*Continued.*

Subject.	Method.	Blood as drawn.	Blood + known amounts of glucose.		
			Addition.	Found.	Calculated.
		per cent		*per cent*	*per cent*
9. A. J., 22 yrs., convalescent.	Bang.	0.097	10 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.162	0.164
	Hagedorn.	0.092		0.150	0.160
	Benedict.	Lost.			
	Folin.	0.111		0.160	0.176
10. E. B., 67 yrs., chronic rheumatism.	Bang.	0.082	10 cc. of blood + 3 cc. of 0.5 per cent glucose solution.	0.177	0.178
	Hagedorn.	0.097		0.185	0.190
	Benedict.	0.095		0.196	0.195
	Folin.	0.098		Lost.	
11. G. V., 25 yrs., rheumatism.	Bang.	0.081	10 cc. of blood + 3 cc. of 0.5 per cent glucose solution.	0.180	0.177
	Hagedorn.	0.092		0.179	0.186
	Benedict.	0.098		0.200	0.190
	Folin.	0.108		Lost.	
12. A. T., 22 yrs., convalescent.	Bang.	0.091	10 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.162	0.160
	Hagedorn.	0.095		0.167	0.163
	Benedict.	0.103		0.175	0.168
	Folin.	0.103		0.186	0.169
13. H., 39 yrs., bronchitis.	Bang.	0.108	8 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.212	0.202
	Hagedorn.	0.107		0.200	0.206
	Benedict.	0.143		0.235	0.237
	Folin.	0.112		0.226	0.211
14. O. A., 54 yrs., aortic aneurism.	Bang.	0.094			
	Hagedorn.	0.096			
	Benedict.	0.094			
	Folin.	0.109			
15. Dr. J., 30 yrs., healthy.	Bang.	0.080	8 cc. of blood + 2 cc. of 1 per cent glucose solution.	0.267	0.264
	Hagedorn.	0.076		0.262	0.261
	Benedict.	0.090		0.280	0.272
	Folin.	0.098		0.286	0.278
16. I. K., 22 yrs., nephritis.	Bang.	Lost.	10 cc. of blood + 2 cc. of 0.5 per cent glucose solution.		
	Hagedorn.	0.110		0.175	0.175
	Benedict.	0.112		0.185	0.177
	Folin.	0.114		0.186	0.178

TABLE I—*Concluded.*

Subject.	Method.	Blood as drawn.	Blood + known amounts of glucose.		
			Addition.	Found.	Calculated.
		per cent		*per cent*	*per cent*
17. R. P., 20 yrs., nephritis.	Bang.	0.074	8 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.163	0.156
	Hagedorn.	0.088		0.167	0.170
	Benedict.	0.100		0.189	0.180
	Folin.	0.095			
18. O. T., 23 yrs., nephritis.	Bang.	0.070	8 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.159	0.156
	Hagedorn.	0.080		0.164	0.164
	Benedict.	0.089		0.180	0.180
	Folin.	0.091		Lost.	
19. M. S., 65 yrs., coma uremicum.	Bang.	0.118	10 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.199	0.198
	Hagedorn.	0.124		0.200	0.204
	Benedict.	0.151		0.230	0.228
	Folin.	0.121		0.216	0.201
20. I. K., 22 yrs., nephritis.	Bang.	0.097	10 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.180	0.179
	Hagedorn.	0.082		0.163	0.165
	Benedict.	0.097		0.174	0.179
	Folin.	0.093		0.179	0.176
21. G. D., 54 yrs., bronchitis.	Bang.	0.092	8 cc. of blood + 2 cc. of 1 per cent glucose solution.	Lost.	
	Hagedorn.	0.082		0.270	0.266
	Benedict.	0.100		0.274	0.280
	Folin.	0.105		0.276	0.284
22. O. A., 38 yrs., pleuritis.	Bang.	0.110			
	Hagedorn.	Lost.			
	Benedict.	0.105			
	Folin.	0.108			
23. M. S., 65 yrs., coma uremicum.	Bang.	Lost.			
	Hagedorn.	0.123			
	Benedict.	0.131			
	Folin.	0.140			

TABLE II.

Diabetic Subjects.

Subject.	Method.	Blood as drawn.	Blood + known amounts of glucose.		
			Addition.	Found.	Calculated.
		per cent		*per cent*	*per cent*
24. A. T., 21 yrs.	Bang.	0.090	10 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.169	0.173
	Hagedorn.	Lost.			
	Benedict.	0.108		0.182	0.189
	Folin.	0.095		0.178	0.177
25. B. L., 35 yrs. Dec. 31.	Bang.	0.283			
	Hagedorn.	0.270			
	Benedict.	0.286			
	Folin.	0.281			
26. B. L., 35 yrs. Jan. 1.	Bang.	0.270			
	Hagedorn.	0.250			
	Benedict.	0.286			
	Folin.	0.278			
27. A. G., 42 yrs. Jan. 3.	Bang.	0.089			
	Hagedorn.	0.088			
	Benedict.	0.103			
	Folin.	0.095			
28. A. G., 42 yrs. Jan. 4.	Bang.	0.096			
	Hagedorn.	0.093			
	Benedict.	0.118			
	Folin.	0.111			
29. B. L., 35 yrs. Jan. 5.	Bang.	0.214			
	Hagedorn.	0.213			
	Benedict.	0.227			
	Folin.	0.218			
30. E. B., 29 yrs.	Bang.	0.065			
	Hagedorn.	0.070			
	Benedict.	0.080			
	Folin.	0.071			
31. K. D., 55 yrs.	Bang.	0.172			
	Hagedorn.	0.181			
	Benedict.	0.208			
	Folin.	0.176			

TABLE II—*Concluded.*

Subject.	Method.	Blood as drawn.	Blood + known amounts of glucose.		
			Addition.	Found.	Calculated.
		per cent		*per cent*	*per cent*
32. A. H., 20 yrs.	Bang.	0.187			
	Hagedorn.	0.191			
	Benedict.	0.222			
	Folin.	0.182			
33. A. H., 20 yrs.	Bang.	0.321	10 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.347	0.351
	Hagedorn.	Lost.			
	Benedict.	0.351		0.390	0.375
	Folin.	0.333		0.345	0.361
34. H. H., 61 yrs. Jan. 9.	Bang.	0.145	10 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.212	0.204
	Hagedorn.	0.159		0.222	0.216
	Benedict.	0.198		0.247	0.248
	Folin.	0.188		0.236	0.240
35. H. H., 61 yrs. Jan. 12.	Bang.	0.101	10 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	Lost.	
	Hagedorn.	0.103		0.163	0.168
	Benedict.	0.150		0.198	0.208
	Folin.	0.121		0.202	0.183
36. H. H., 61 yrs.	Bang.	0.101			
	Hagedorn.	0.121			
	Benedict.	0.150			
	Folin.	0.135			
37. A. H., 31 yrs.	Bang.	0.151	8 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.216	0.221
	Hagedorn.	0.171		0.240	0.237
	Benedict.	0.166		0.230	0.233
	Folin.	0.195		0.266	0.256

TABLE III.

Method.	Average error.	Greatest error.	No. of analyses.
	per cent	*per cent*	
Bang	2.7	8	20
Hagedorn	3.2	9	22
Benedict	3.0	7	22
Folin	4.5	10	19

The highest limit for blood sugar concentration in a fasting condition is, as is known, of importance for deciding whether hyperglycemia is present. Bang set the limit at 0.12 per cent by his old method, but later, after he had modified his method, he set the limit at 0.11 per cent.

In our nine normal individuals and eight non-diabetics, who were all examined in a fasting state in the morning, we find, leaving out of account Analyses 19 and 23 from a patient with coma uremicum, the blood sugar values determined by Bang's method and by Hagedorn's method always lie under 0.11 per cent. Folin's method twice gives values about 0.11 per cent, otherwise the results by this method also lie under 0.11 per cent. Benedict's method, on the other hand, three times gives values between 0.11 and 0.12 per cent, and once even a value of 0.143 per cent.

SUMMARY.

It is uncertain whether any of the blood sugar methods hitherto used give only the amount of glucose. Bang and Hatlehoel's method and Hagedorn and Jensen's method give in normal individuals and in diabetics values which approximately agree. Folin and Wu's method as well as Myers and Bailey's (10) modification of Lewis and Benedict's method, may, at any rate in diabetics with hyperglycemia, give too high results. This is especially the case with the last mentioned method.

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10. Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

NOTE ON THE FAT-SOLUBLE GROWTH-PROMOTING SUBSTANCE IN LARD AND COTTON-SEED OIL.

BY AMY L. DANIELS AND ROSEMARY LOUGHLIN.

(*From the Department of Nutrition, Child Welfare Research Station, University of Iowa, Iowa City.*)

(Received for publication, April 28, 1920.)

It is generally conceded by workers in the field of nutrition that the fat-soluble growth-promoting substance in certain fats, more particularly the extracted vegetable oils and lard, is present in such low concentration, if at all, that from the standpoint of nutrition these fats are of value merely as sources of energy. Indeed, there are reports in the literature of many feeding experiments where nutritive disaster on diets, which included vegetable oils or lard in fairly liberal amounts, have been averted by the substitution or addition of small amounts of those fats—butter fat, egg yolk fat, or cod liver oil—which are known to be rich in the fat-soluble vitamine.[1] The results with the vegetable oils and lard are not in all cases in accord with those which we have obtained in our laboratory; and we are led to believe that there may be some difference in the amount of the fat-soluble complex in commercial lards, and that certain vegetable oils may have values other than that of furnishing energy.

Since the relation between the fat-soluble vitamine and rickets is undergoing experimental scrutiny, our findings may be of interest in this connection. It is possible that recent workers[2] who have been using the vegetable oils, more particularly cotton-seed, in the study of rickets, with the thought that these are quite free from the fat-soluble complex may have been furnishing enough of the vitamine to meet the requirements of growth, especially if the experimental periods were of comparatively short duration.

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[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913–14, xvi, 423; 1915, xx, 379; 1918, xxxiv, 17. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1913, xv, 167; 1915, xxi, 179.

[2] Hess, A. F., *J. Biol. Chem.*, 1920, xli, p. xxxii.

In an investigation dealing with some problems in infant nutrition we had occasion to feed groups of rats on purified rations in composition simulating milk but which, we believed, contained the least possible amount of the fat-soluble hormone. These rations consisted of 18 per cent of casein, 28 per cent of fat, both lard and cotton-seed, respectively, 7 per cent of our salt mixture, 47 per cent of corn-starch, and the water-alcohol extract of 9 gm. of wheat embryo (cold process). In order to make sure that our experimental rations were as free as possible from the fat-soluble complex, both the casein and the wheat embryo, previous to the alcohol treatment, were extracted for 48 hours with ether ("Squibb, for anæsthesia") in a Soxhlet apparatus. The lard used was a commercial product rendered from the leaves and back fat. The cotton-seed oil was also a commercial product.[3]

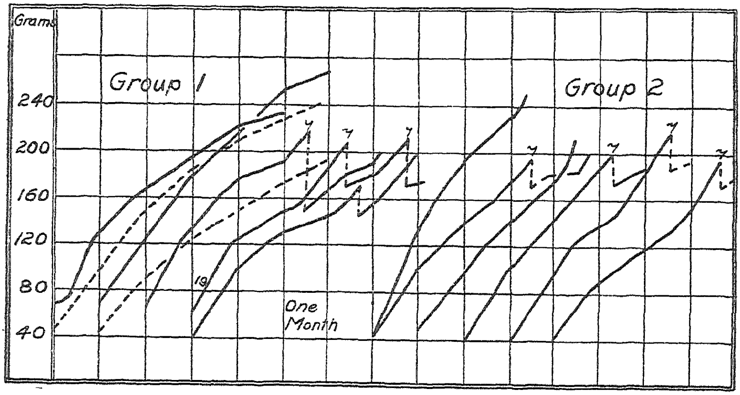

CHART 1. Both lard and cotton-seed oil apparently contain an appreciable amount of the fat-soluble vitamine. Animals fed rations consisting of casein 18 per cent, corn-starch 47 per cent, a suitable salt mixture, and 28 per cent of lard (Group 1) or cotton-seed oil (Group 2), respectively, together with an adequate amount of the water-alcohol extract of wheat embryo, grew normally, reproduced, and reared their young.

Our animals on these rations have grown normally; in fact, their growth curves (Chart 1) are quite similar to those of control animals fed a purified ration which included 5 per cent of butter fat in place of an equivalent amount of lard or cotton-seed oil, and in which neither casein nor the wheat embryo was extracted (Chart 2). All females in both lard and cotton-seed groups have reproduced and in certain instances second litters have been obtained. The young in both groups have been successfully reared on the diets of their mothers.

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[3] The cotton-seed oil was a product of the American Cotton Oil Company, New York.

These results were so unexpected and so out of harmony with the published results of other workers that it was deemed of interest to determine the effects of a somewhat less amount of these two fats. Therefore rations in all other respects the same, but containing 53 per cent of corn-starch and only 21 per cent of the fats under investigation, were fed. Since the food value of this mixture was 4.77 calories per gm. whereas that of our first mixture furnished 5.12 calories per gm., it was reasoned that the animals on this second mixture were getting about 6 per cent less fat than those receiving the 28 per cent fat ration.

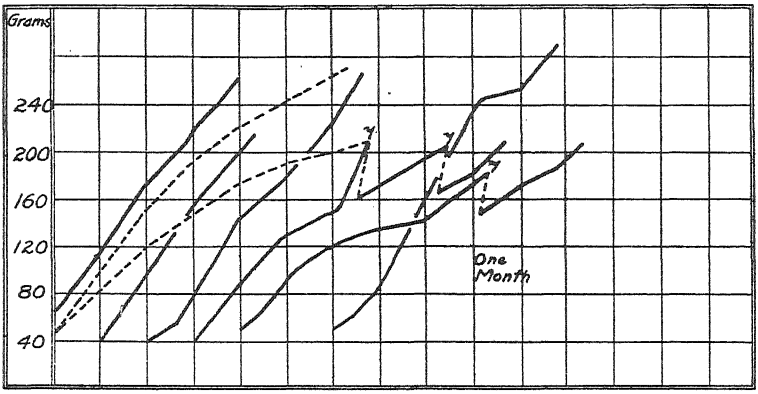

Chart 2. Curves of growth of control animals fed purified rations in which 5 per cent of butter was substituted for an equivalent amount of lard.

On the lower fat food all the animals fed the lard mixture made normal growth gains for about 2 months, when the weight became stationary (Chart 3, Group 1). Those rats receiving the ration containing the lesser amount of cotton-seed oil (21 per cent) also grew far less well than those on the higher fat, the more vigorous animals growing normally for only about 6 weeks, when there was a gradual decline in weight (Chart 3, Group 2).

These latter results are in accord with those of previous workers who have shown that both lard and cotton-seed oil contain much less of the fat-soluble vitamine than certain other fats. But, considering the fact that animals have made normal growth, reproduced, and reared their young on diets in which the only apparent source of the vitamine was the lard or cotton-seed oil, it would seem that both these fats contain appreciable amounts of the fat-soluble growth stimulant. This, however, is demonstrable only when fairly large amounts are fed.

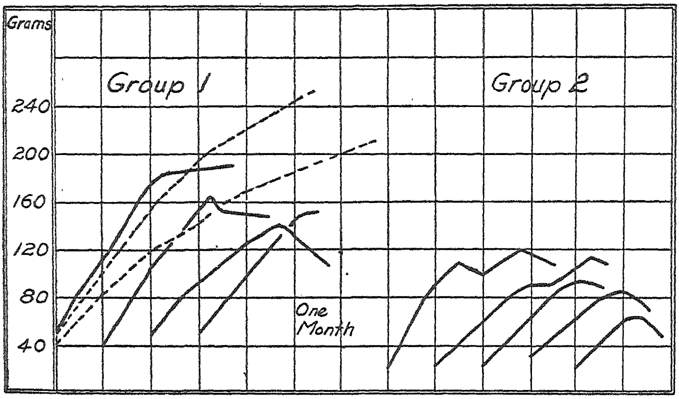

CHART 3. Animals fed purified rations furnishing 21 per cent of lard (Group 1) and cotton-seed oil (Group 2), respectively, failed to grow normally. Slightly better results were obtained with the lard than with the cotton-seed oil rations.

ACIDITY OF ASH-FREE AND OF COMMERCIAL GELATIN SOLUTIONS.

BY H. E. PATTEN AND T. O. KELLEMS.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

(Received for publication, May 4, 1920.)

In connection with measurements of the hydrogen ion concentration of various gelatin solutions,[1] it was thought desirable to compare the free acidity of ash-free gelatin with that of commercial gelatin. Accordingly, solutions were made up containing 0.5 per cent of ash-free[2] gelatin and different concentrations of hydrochloric acid (or of sodium hydroxide) and their hydrogen ion concentrations determined in each case electrometrically. The results obtained are given in Table I.

In this table the first column shows the normality of the gelatin solutions with respect to hydrochloric acid (or to sodium hydroxide). The second column shows the pH value.[3]

Similarly, solutions containing 0.5 per cent of commercial gelatin were made up, the acid alkali range of concentration being the same as for the solutions of ash-free gelatin, and the hydrogen ion concentration was measured. The results are given in Table II.

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[1] Patten, H. E., and Johnson, A. J., *J. Biol. Chem.*, 1919, xxxviii, 179.

[2] The ash-free gelatin used in these experiments was kindly furnished by Mr. C. R. Smith of the Bureau of Chemistry.

[3] The hydrogen ion concentration was measured by a Clark electrode vessel, our electrode being of gold plated with paladium black immediately before each determination. The voltage was read on a Leeds and Northrup potentiometer of standard design, the temperature of the electrode cell and of the calomel half cell being maintained by an air thermostat, electrically controlled, at 25°C.

$$\text{pH} = \log \frac{1}{\text{concentration of hydrogen ion}}$$

Thus, a solution of 0.01 N with respect to hydrogen ions would have a pH = 2.

The results are presented graphically in Fig. 1. The exponent of the hydrogen ion concentration, pH, is taken as abscissa and the normality of the total system with respect to acid (or to alkali) as ordinate. To avoid excessive use of ciphers the negative exponent of the normality is used; thus, where the normality is 1×10^{-6} 6 is used. 1 does not mean normal but 0.1 normal. The ordinate thus extends from the highest concentration of acid through zero concentration of acid on through the regions of higher alkaline concentration.

TABLE I.

Normality with respect to HCl.	pH	Normality with respect to NaOH.	pH
10^{-1}	0.77	0.0	4.81
10^{-2}	1.96	10^{-6}	4.89
10^{-3}	3.89	10^{-5}	5.00
10^{-4}	4.69	10^{-4}	5.06
10^{-5}	4.74	10^{-3}	6.49
10^{-6}	4.83	10^{-2}	11.33

TABLE II.

Normality with respect to HCl.	pH	Normality with respect to NaOH.	pH
10^{-1}	0.96	0.0	5.6
10^{-2}	2.12	10^{-6}	5.54
10^{-3}	4.49	10^{-5}	5.66
10^{-4}	5.4	10^{-4}	5.69
10^{-5}	5.6	10^{-3}	8.78
10^{-6}	5.6	10^{-2}	11.57

It will be observed that the isoelectric point of ash-free gelatin (Smith), Curve 1, lies at pH = 4.8, corresponding to a hydrogen ion concentration of 1.59×10^{-5}, and that the isoelectric point of commercial gelatin, Curve 2, lies further toward the alkaline region and comes at pH = 5.64 (hydrogen ion concentration of 2.28×10^{-5}).

Ash determinations were made on both samples of gelatin used. The ash-free gelatin (Smith) showed on ignition 0.011 per cent residue which on treatment with hydrofluoric acid (to remove silica) and after a second ignition was completely volatilized. A second portion of the gelatin was ignited and, without using hydrofluoric acid, the ash was treated with neutral distilled water. This solution showed no alkalinity with phenol red. The sample of commercial gelatin, however, showed a residue after the first ignition of 0.74 per cent. After being treated eight times with hydrofluoric acid this residue was reduced to 0.25 per cent of the original gelatin. A second portion of the commercial gelatin was ignited and the ash dissolved in neutral distilled water and titrated with standard HCl solution. This

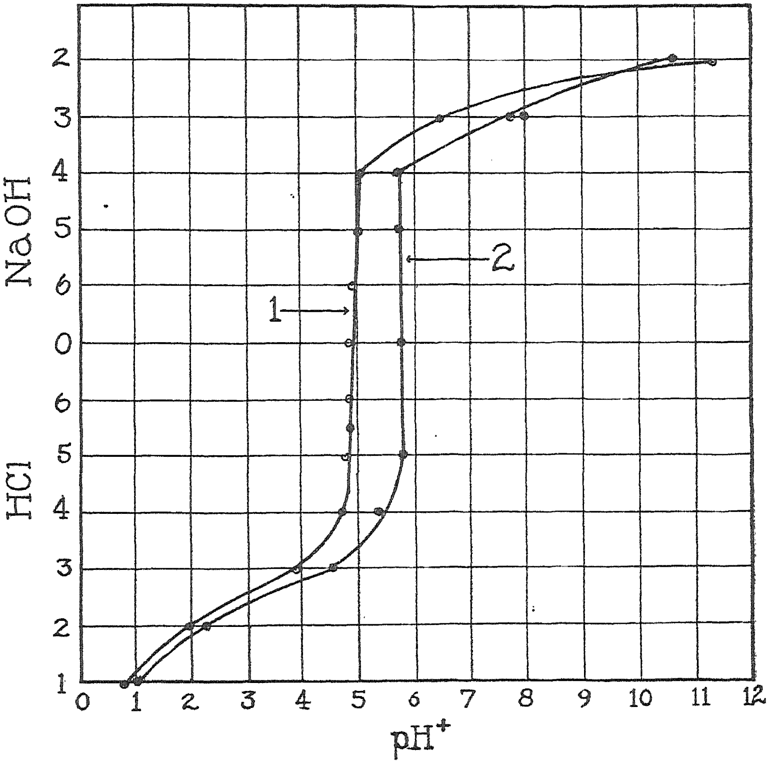

Fig. 1. Titration of 0.5 per cent gelatin in water at 25°C. Curve 1, ash-free gelatin (Smith). Curve 2, commercial gelatin. Ordinates, normality of acid (or alkali) in total system expressed as the negative index of normality; abscissæ are pH $^{+}$.

showed an alkalinity corresponding to 0.000577 gm. of CaO per gm. of gelatin, or to a pH of 4.29 in a 0.5 per cent gelatin solution (hydrogen ion concentration of 5.14×10^{-5}), assuming it is all in solution.

It is evident that the displacement of the isoelectric point of commercial gelatin was due to the alkali content, part of the alkali being combined with the gelatin so that not all the alkali appearing in the ash is available to displace the hydrogen ion concentration.

The isoelectric point of ash-free gelatin (Smith) is identical with that of Loeb's so called isoelectric gelatin.[4]

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[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

SEDOHEPTOSE, A NEW SUGAR FROM SEDUM SPECTABILE. II.

By F. B. La FORGE.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

(Received for publication, March 30, 1920.)

In a previous publication[1] a new ketose was described possessing properties which seemed to place it in a unique position among all known members of the sugar group.

While the original compound reduced Fehling's solution strongly, it lost about 80 per cent of its reducing power on boiling with dilute acids.

When the transformed compound was treated with benzaldehyde in 50 per cent H_2SO_4 solution, a crystalline product was obtained, which proved to be the benzal derivative of a heptose that had lost 1 molecule of water. By hydrolysis of this benzal compound the anhydride itself ($C_7H_{12}O_6$) was obtained as crystalline anhydrosedoheptose. This compound did not reduce Fehling's solution. On heating with dilute acid the anhydride passed to the extent of about 20 per cent into the reducing sedoheptose ($C_7H_{14}O_7$) and there is thus an equilibrium in such solutions between the two substances.

Upon reduction with sodium amalgam in neutral or slightly alkaline solution sedoheptose yielded two heptitols, which property, and also the fact that it was not oxidized by bromine, served to characterize the new sugar as a ketose.

Its tendency to form a non-reducing anhydride, which may be regarded also as an inner glucoside, is a property which served to differentiate the sugar of the sedum plant from all other members of this group.

There are several considerations of structure which might offer some explanation of the abnormality displayed by sedoheptose. One of these is the theory that sedoheptose might have a branch chain formula such as

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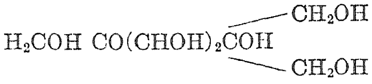
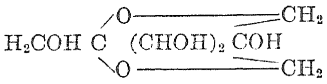
[1] La Forge, F. B., and Hudson, C. S., *J. Biol. Chem.*, 1917, xxx, 61.



$$H_2COH\ CO(CHOH)_2COH\begin{matrix} CH_2OH \\ CH_2OH \end{matrix}$$

The anhydride might then be represented by



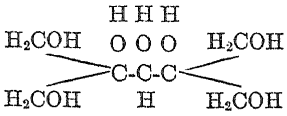
But there are two good reasons why the branch chain theory must be excluded.

In the first place, one of the heptitols obtained on reduction of sedoheptose is inactive, while all possible branch chain heptitols, according to theoretical considerations, are active.[2] Second, oxidation of a branch chain heptitol with HNO_3 should lead to a tribasic acid, while experiment showed that a dibasic, pentoxypimelic acid was obtained on oxidation of sedoheptitol.

Oxidation of α-Sedoheptitol.

18 gm. of α-sedoheptitol[1] were oxidized according to the method of Fischer by warming with three parts of HNO_3 (specific gravity 1.2). After standing for 3 or 4 hours at about 50° the solution was rapidly concentrated on the steam bath and most of the excess acid expelled by repeated evaporation with water. The reaction product was finally boiled with calcium carbonate for several hours and filtered. The filtrate slowly deposited crystals which were collected and purified by dissolving in water containing a slight excess of oxalic acid and again transforming the acid into the calcium salt. The solution yielded the salt as pure white crystals. The yield was 3 gm.

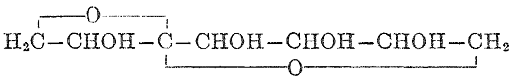
[2] It is, to be sure, possible to write a formula for an inactive heptitol having no asymmetric carbon atoms, thus:



0.1898 gm. substance dried at 100° gave 0.2132 gm. CO_2, 0.0539 gm. H_2O, and 0.0372 gm. CaO.

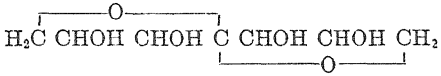
	Calculated for Ca $C_7H_{10}O_9$ (278).	Found.
	per cent	*per cent*
Ca...................................	14.38	14.00
C...................................	30.21	30.60
H...................................	3.59	3.15

These results agree for the Ca salt of pentoxypimelic acid. Therefore, sedoheptitol and, consequently, sedoheptose in all probability have a straight chain formula. The three possible structures for straight chain, seven carbon ketoses are: alpha, CH_2OH CO CHOH CHOH CHOH CHOH CH_2OH; beta, CH_2OH CHOH CO CHOH CHOH CHOH CHOH CH_2OH; and gamma,[3] CH_2OH CHOH CHOH CO CHOH CHOH CH_2OH. Of these three possibilities beta and gamma might explain the formation of an anhydride as follows. Beta (by elimination of water)



$$\mathrm{H_2C{-}CHOH{-}C{-}CHOH{-}CHOH{-}CHOH{-}CH_2}$$

and gamma (by elimination of water)



$$\mathrm{H_2C\ CHOH\ CHOH\ C\ CHOH\ CHOH\ CH_2}$$

As has already been stated two heptitols have been obtained from sedoheptose by reduction; one, α-sedoheptitol, is active, the other, β-sedoheptitol, is inactive.

It will readily be seen that if the space formulas of these heptitols were known the configuration and also the structure of sedoheptose would follow, because the two heptitols would differ only in having the position of one hydroxyl reversed. The carbon atom on which this reversal took place would be the one which originally carried the carbonyl group in the ketose from which the two heptitols were derived. Thus, in the case of fructose,

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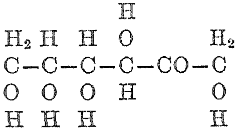
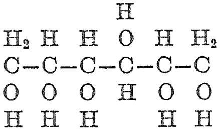
[3] The words alpha, beta, and gamma are written out where they refer to the position of carbon atoms in the molecule. The Greek letters α and β are used to distinguish between epimeric compounds.



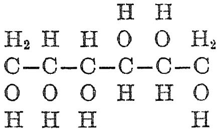
$$\begin{array}{ccccccccccc} & & & & & & \mathrm{H} & & & & \\ \mathrm{H_2} & & \mathrm{H} & & \mathrm{H} & & \mathrm{O} & & & & \mathrm{H_2} \\ \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{CO} & - & \mathrm{C} \\ \mathrm{O} & & \mathrm{O} & & \mathrm{O} & & \mathrm{H} & & & & \mathrm{O} \\ \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & & & & & \mathrm{H} \end{array}$$

the two resulting hexitols, sorbitol



$$\begin{array}{ccccccccccc} & & & & & & \mathrm{H} & & & & \\ \mathrm{H_2} & & \mathrm{H} & & \mathrm{H} & & \mathrm{O} & & \mathrm{H} & & \mathrm{H_2} \\ \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} \\ \mathrm{O} & & \mathrm{O} & & \mathrm{O} & & \mathrm{H} & & \mathrm{O} & & \mathrm{O} \\ \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & & & \mathrm{H} & & \mathrm{H} \end{array}$$

and mannitol



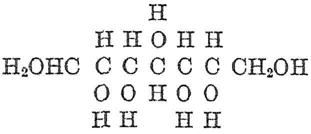
$$\begin{array}{ccccccccccc} & & & & & & \mathrm{H} & & \mathrm{H} & & \\ \mathrm{H_2} & & \mathrm{H} & & \mathrm{H} & & \mathrm{O} & & \mathrm{O} & & \mathrm{H_2} \\ \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} \\ \mathrm{O} & & \mathrm{O} & & \mathrm{O} & & \mathrm{H} & & \mathrm{H} & & \mathrm{O} \\ \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & & & & & \mathrm{H} \end{array}$$

have the hydroxyl groups reversed in the alpha position and the formula of the sugar would be evident if only the configuration of sorbitol and mannitol were known. The same method could be applied to sedoheptose.

There are according to the theory ten heptitols, if optical isomerism is disregarded. Six of these are optically active and four are inactive. Their space formulas are as follows.

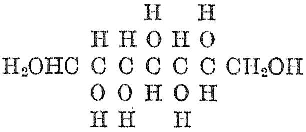
Heptitols.

From Glucose.



$$\begin{array}{ccccccc} & & & \mathrm{H} & & & \\ & \mathrm{H} & \mathrm{H} & \mathrm{O} & \mathrm{H} & \mathrm{H} & \\ \mathrm{H_2OHC} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{CH_2OH} \\ & \mathrm{O} & \mathrm{O} & \mathrm{H} & \mathrm{O} & \mathrm{O} & \\ & \mathrm{H} & \mathrm{H} & & \mathrm{H} & \mathrm{H} & \end{array}$$

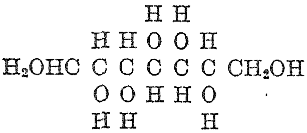
1*i*



$$\begin{array}{ccccccc} & & & \mathrm{H} & & \mathrm{H} & \\ & \mathrm{H} & \mathrm{H} & \mathrm{O} & \mathrm{H} & \mathrm{O} & \\ \mathrm{H_2OHC} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{CH_2OH} \\ & \mathrm{O} & \mathrm{O} & \mathrm{H} & \mathrm{O} & \mathrm{H} & \\ & \mathrm{H} & \mathrm{H} & & \mathrm{H} & & \end{array}$$

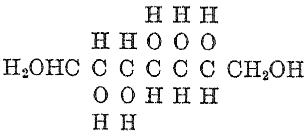
2*a*

From Mannose.



$$\begin{array}{ccccccc} & & & \mathrm{H} & \mathrm{H} & & \\ & \mathrm{H} & \mathrm{H} & \mathrm{O} & \mathrm{O} & \mathrm{H} & \\ \mathrm{H_2OHC} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{CH_2OH} \\ & \mathrm{O} & \mathrm{O} & \mathrm{H} & \mathrm{H} & \mathrm{O} & \\ & \mathrm{H} & \mathrm{H} & & & \mathrm{H} & \end{array}$$

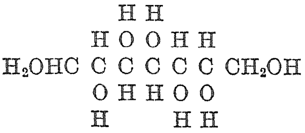
3*a*



$$\begin{array}{ccccccc} & & & \mathrm{H} & \mathrm{H} & \mathrm{H} & \\ & \mathrm{H} & \mathrm{H} & \mathrm{O} & \mathrm{O} & \mathrm{O} & \\ \mathrm{H_2OHC} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{C} & \mathrm{CH_2OH} \\ & \mathrm{O} & \mathrm{O} & \mathrm{H} & \mathrm{H} & \mathrm{H} & \\ & \mathrm{H} & \mathrm{H} & & & & \end{array}$$

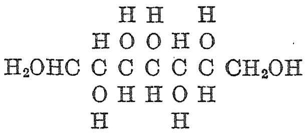
4*a*

From Galactose.



$$\begin{array}{rcccccl} & & H & H & & & \\ & H & O & O & H & H & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & H & H & O & O & \\ & H & & & H & H & \end{array}$$

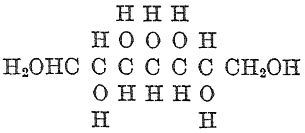
3*a*



$$\begin{array}{rcccccl} & & H & H & & H & \\ & H & O & O & H & O & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & H & H & O & H & \\ & H & & & H & & \end{array}$$

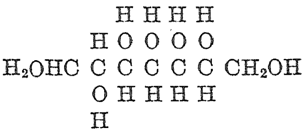
5*a*

From Talose.



$$\begin{array}{rcccccl} & & H & H & H & & \\ & H & O & O & O & H & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & H & H & H & O & \\ & H & & & & H & \end{array}$$

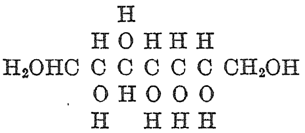
6*i*



$$\begin{array}{rcccccl} & & H & H & H & H & \\ & H & O & O & O & O & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & H & H & H & H & \\ & H & & & & & \end{array}$$

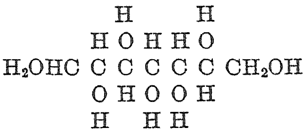
7*a*

From Gulose.



$$\begin{array}{rcccccl} & & H & & & & \\ & H & O & H & H & H & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & H & O & O & O & \\ & H & & H & H & H & \end{array}$$

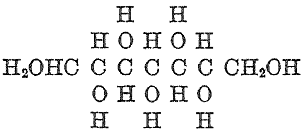
8*a*



$$\begin{array}{rcccccl} & & H & & & H & \\ & H & O & H & H & O & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & H & O & O & H & \\ & H & & H & H & & \end{array}$$

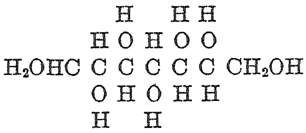
5*a*

From Idose.



$$\begin{array}{rcccccl} & & H & & H & & \\ & H & O & H & O & H & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & H & O & H & O & \\ & H & & H & & H & \end{array}$$

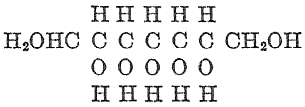
9*i*



$$\begin{array}{rcccccl} & & H & & H & H & \\ & H & O & H & O & O & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & H & O & H & H & \\ & H & & H & & & \end{array}$$

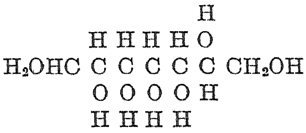
2*a*

From Allose.



$$\begin{array}{rcccccl} & H & H & H & H & H & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & O & O & O & O & \\ & H & H & H & H & H & \end{array}$$

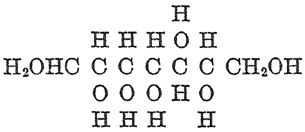
10*i*



$$\begin{array}{rcccccl} & & & & & H & \\ & H & H & H & H & O & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & O & O & O & H & \\ & H & H & H & H & & \end{array}$$

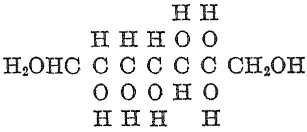
7*a*

From Altrose.



$$\begin{array}{rcccccl} & & & & H & & \\ & H & H & H & O & H & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & O & O & H & O & \\ & H & H & H & & H & \end{array}$$

8*a*



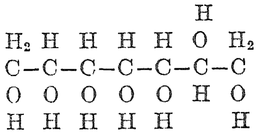
$$\begin{array}{rcccccl} & & & & H & H & \\ & H & H & H & O & O & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & O & O & H & O & \\ & H & H & H & & H & \end{array}$$

4*a*

Those which are active are designated *a*, those which are inactive, *i*. Of the active heptitols all but 7*a* are known while 1*i* is the only known inactive one.

Neither of the heptitols from sedoheptose is identical with any of the known heptitols. But since one of them, α-sedoheptitol, is active it must have the formula

7*a*



$$\begin{array}{ccccccccccccc} & & & & & & & & & & \mathrm{H} & & \\ \mathrm{H_2} & & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \mathrm{O} & & \mathrm{H_2} \\ \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} \\ \mathrm{O} & & \mathrm{O} & & \mathrm{O} & & \mathrm{O} & & \mathrm{O} & & \mathrm{H} & & \mathrm{O} \\ \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & & & \mathrm{H} \end{array}$$

because this is the only remaining possibility according to the theory.

The inactive β-sedoheptitol might have one of three possible configurations, 6*i*, 9*i*, or 10*i*, since 1*i* is known and is excluded on account of the properties of its acetyl and benzal derivatives.[4]

Returning to the consideration of the three possible structures, alpha, beta, and gamma of sedoheptose, we may exclude the last named possibility for the reason that the gamma position in the case of a seven carbon chain is in the middle of the molecule. From this it follows that if the keto group in the original sugar was in this position, and if it yielded on reduction two heptitols, one of which was inactive, the second heptitol which resulted from this treatment would also have to be inactive. Therefore, the gamma keto structure is excluded.

There remain the alpha and beta structures to be considered.

The latter structure might explain the abnormal properties of sedoheptose, but the properties of α- and β-sedoheptitol are not in agreement with theoretical requirements. Inactive β-sedoheptitol must have one of the configurations 6*i*, 9*i*, or 10*i* and α-sedoheptitol must differ in having the position of one hydroxyl in the beta position reversed. With this change 1*i* and 6*i* would yield 3*a* or α-mannoheptitol of M. P. 180° and could not be confused with active α-sedoheptitol. Likewise, with the same change both 9*i* and 10*i* would yield 8*a* (β-guloheptitol of M. P.

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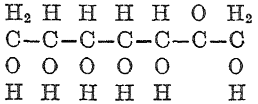
[4] Fischer, E., *Ann. Chem.*, 1892, cclxx, 64. Wherry, E. T., *J. Biol. Chem.*, 1920, xlii, 377.

128°).[5] Thus, the beta possibility must also be excluded and there remains only the alpha structure to be considered.

There are two possible configurations for an alpha keto structure which will meet the conditions in the case of sedoheptose.

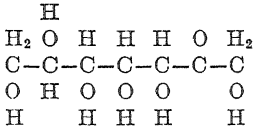
I.



$$\begin{array}{ccccccccccccc} H_2 & & H & & H & & H & & H & & O & & H_2 \\ C & - & C & - & C & - & C & - & C & - & C & - & C \\ O & & O & & O & & O & & O & & & & O \\ H & & H & & H & & H & & H & & & & H \end{array}$$

and

II.



$$\begin{array}{ccccccccccccc} & & H & & & & & & & & & & \\ H_2 & & O & & H & & H & & H & & O & & H_2 \\ C & - & C & - & C & - & C & - & C & - & C & - & C \\ O & & H & & O & & O & & O & & & & O \\ H & & & & H & & H & & H & & & & H \end{array}$$

---

[5] La Forge, F. B., *J. Biol. Chem.*, 1920, xli, 251.

β-Glucoheptitol 2*a* and β-guloheptitol 8*a* both melt at about the same temperature as β-sedoheptitol and all three show little or no optical activity. It is not possible, however, that β-sedoheptitol could be identical with either, as can be shown by theoretical as well as experimental considerations. The space formulas on pages 370 and 371 will show that neither 2*a* nor 8*a* could lead to 7*a*, the only remaining unknown active heptitol by the reversal of the position of one hydroxyl group.

In the following table the melting points of the three compounds in question are given together with their optical rotations.

	Melting point. °C.	$[\alpha]_D$
β-Glucoheptitol	130–131	+0.8
β-Guloheptitol	129	0
β-Sedoheptitol[1]	128	0
β-Gluco- + β-sedoheptitol	119–120	
β-Gulo- + β-sedoheptitol	121	
β-Gluco- + β-guloheptitol	118–120	

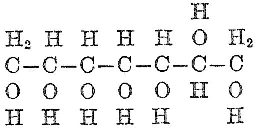
In addition to these differences β-glucoheptitol forms a characteristic gelatinous benzal derivative. Dr. Edgar T. Wherry of this Bureau has measured the refractive indices of the three substances with the following results.

	α	β	γ	γ-α
β-Glucoheptitol	1.542	1.550	1.552	0.010
β-Guloheptitol	1.565	1.570	1.586	0.021
β-Sedoheptitol	1.564	1.570	1.584	0.020

The differences brought out between β-glucoheptitol serve to differentiate it sharply from the other two. The figures for β-guloheptose and β-sedoheptose, however, do not show sufficient differences to allow a conclusion to be drawn as to their separate identity. This result is not at all surprising since the last mentioned compounds differ only in the configuration of their molecules by the position of one hydroxyl group.

Both of these configurations will give on reduction the active heptitol

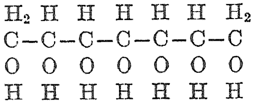
7*a*



$$\begin{array}{ccccccc} & & & & & H & \\ H_2 & H & H & H & H & O & H_2 \\ C- & C- & C- & C- & C- & C- & C \\ O & O & O & O & O & H & O \\ H & H & H & H & H & & H \end{array}$$

and both will give unknown inactive heptitols

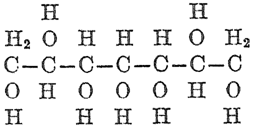
10*i*



$$\begin{array}{ccccccc} H_2 & H & H & H & H & H & H_2 \\ C- & C- & C- & C- & C- & C- & C \\ O & O & O & O & O & O & O \\ H & H & H & H & H & H & H \end{array}$$

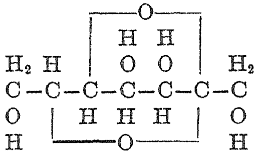
in the case of I and

6*i*



$$\begin{array}{ccccccc} & H & & & & H & \\ H_2 & O & H & H & H & O & H_2 \\ C- & C- & C- & C- & C- & C- & C \\ O & H & O & O & O & H & O \\ H & & H & H & H & & H \end{array}$$

in the case of II. Any two of the five hydroxyl groups might be involved in the formation of the anhydride, which might have, for instance, the formula



$$\begin{array}{ccccccc} & & & O & & & \\ & & & H & H & & \\ H_2 & H & & O & O & & H_2 \\ C- & C- & C- & C- & C- & C- & C \\ O & & H & H & H & & O \\ H & & & O & & & H \end{array}$$

It is not possible with our present knowledge to decide between I and II and before this question can be settled the active heptitol, 7*a*, and one of the inactive ones, 10*i* or 9*i*, will have to be prepared. For the preparation of these compounds considerable amounts of talose or allose would be necessary, and until this material is available the decision between the two possible formulas for sedoheptose will have to be withheld.

The formulas for sedoheptose, one of which one seems forced to accept, do not offer any explanation for the abnormal behavior of this sugar toward dilute acids. The only peculiarity in the case of I is in the hydroxyl groups, all being on one side of the molecule. Whether such a circumstance can, in general, be the cause of such a great difference in properties only further study of the higher sugars can decide.

VOLEMITE.

By F. B. La FORGE.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

(Received for publication, January 21, 1920.)

In a previous article on sedoheptose[1] reference was made to the natural heptitol, volemite. This compound seemed to agree in its properties with those of α-sedoheptitol, one of the heptitols obtained on reduction of sedoheptose with sodium amalgam. The identity of the two compounds would have been accepted but for a discrepancy in the melting points of tribenzal α-sedoheptitol and that recorded by Bougault and Allard[2,3] for the corresponding derivative of volemite.

Since the publication of the article on sedoheptose, I have received, through the courtesy of Professor Emile Bourquelot of Paris, a sample of natural volemite and also a statement that the recorded melting point of tribenzal volemite was erroneous.

The sample furnished by Professor Bourquelot, after crystallization from 75 per cent alcohol, melted at 151° uncorrected. This is the melting point which has been accepted for α-sedoheptitol. The two products, when mixed, also melted at 151° uncorrected.

An optical examination of crystals of the two substances failed to disclose any differences.

For further comparison, the acetal of α-sedoheptitol has been prepared.

Acetal of α-sedoheptitol.—1.4 gm. of α-sedoheptitol in 3 cc. of 50 per cent sulfuric acid were shaken with 3 gm. of acetaldehyde and the reaction mixture was allowed to stand over night in the ice box. The resulting mass of crystals was washed with water and twice recrystallized from a small amount of 75 per cent alcohol. The compound melted at 191–194° uncorrected which agrees with the melting point of volemite acetal recorded by Bougault and Allard.[2] 0.5544 gm. in 25 cc. of chloroform rotated in a 2 dm. tube $-2.60°$ to the left $(\alpha)_D = -45.55$. The authors record $(\alpha)_D$ for the volemite derivative as -46.40 in chloroform solution. There can, therefore, be no further doubt as to the identity of the two heptitols.

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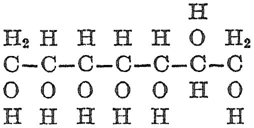
[1] La Forge, F. B., and Hudson, C. S., *J. Biol. Chem.*, 1917, xxx, 61.

[2] Bougault, J., and Allard, G., *Compt. rend. Acad.*, 1902, cxxxv, 796.

[3] In general the benzal derivatives of the higher alcohols are not well adapted for identification purposes as they often show little difference in melting point. In the article on sedoheptose the melting point of tribenzal sedoheptitol was given as 190°. This should be corrected to 225°.

As shown in the preceding publication,[4] the space formula



$$\begin{array}{ccccccccccccc} & & & & & & & & & & \mathrm{H} & & \\ \mathrm{H_2} & & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \mathrm{O} & & \mathrm{H_2} \\ \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} \\ \mathrm{O} & & \mathrm{O} & & \mathrm{O} & & \mathrm{O} & & \mathrm{O} & & \mathrm{H} & & \mathrm{O} \\ \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & \mathrm{H} & & & & \mathrm{H} \end{array}$$

or its mirror image must be accepted as corresponding to α-sedoheptitol and hence must also represent the configuration of volemite.

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[4] La Forge, F. B., *J. Biol. Chem.*, 1920, xlii, 367.

OPTICAL PROPERTIES OF A SERIES OF HEPTITOLS.

By EDGAR T. WHERRY.

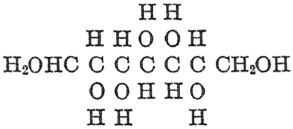
(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

(Received for publication, May 5, 1920.)

Eight of the ten possible heptitols were prepared by Dr. F. B. La Forge of the Bureau of Chemistry and turned over to the writer for investigation.

Optical studies were made of them by the immersion method under the polarizing microscope, using oily immersion liquids of known refractive indices, and light transmitted through a combination of two color screens with a maximum transmission at D. As it seemed of interest to obtain the refractivities, determinations of specific gravity were also made, by suspending the materials in mixtures of carbon tetrachloride and benzene, and measuring the specific gravity of the liquid in each case with a Westphal balance. The n^2 formula[1] was used for calculating refractivity; the theoretical value, using Eisenlohr's data, C, 2.418, H, 1.100, and O, 1.525, should be, for $C_7H_{16}O_7$, 45.20. The results of these observations are here presented.

α-mannoheptitol



$$\begin{array}{ccccccc} & & & H & H & & \\ & H & H & O & O & H & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & O & H & H & O & \\ & H & H & & & H & \end{array}$$

M.P. 180°.

Described by Maquenne.[2]

In ordinary light: seen to consist of minute needles and rods. Refractive indices (D):

$\alpha = 1.538$, $\beta = 1.545$, $\gamma = 1.549$, $\gamma - \alpha = 0.011$, all $\pm$ 0.005; index α is often shown lengthwise. *In parallel polarized light, nicols*

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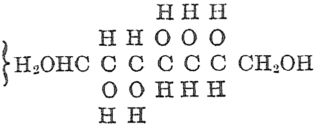
[1] $M = \frac{n^2 - 1}{n^2 + 2} \cdot \frac{W}{\rho}$, where n is the mean refractive index for D, W, the molecular weight (212.13), and ρ, the density of the substance.

[2] Maquenne, *Compt. rend. Acad.*, 1888, cvii, 583.

crossed: Birefringence moderate, the colors being first order; extinction parallel; elongation −.

Specific gravity = 1.485; mean n = 1.544; refractivity = 45.10.

β-mannoheptitol }
α- or β-altroheptitol }



$$\begin{array}{ccccccc} & & & H & H & H & \\ & H & H & O & O & O & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & O & H & H & H & \\ & H & H & & & & \end{array}$$

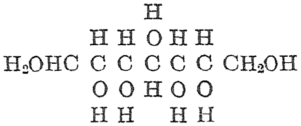
M.P. 125°.

Described by Peirce;[3] prepared from mannose.

In ordinary light: seen to consist of minute needles, often radiating. Refractive indices (D):
$\alpha = 1.533$, $\beta = (?)$, $\gamma = 1.545$, $\gamma - \alpha = 0.012$, all ± 0.005; index γ is often shown lengthwise. *In parallel polarized light, nicols crossed:* Birefringence moderate, the colors being first order; extinction parallel; elongation +.

Specific gravity = 1.470; mean n = 1.539; refractivity = 45.21.

α-glucoheptitol



$$\begin{array}{ccccccc} & & & H & & & \\ & H & H & O & H & H & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & O & H & O & O & \\ & H & H & & H & H & \end{array}$$

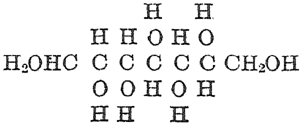
M.P. 128°.

Described by Fischer.[4]

In ordinary light: seen to consist of pointed rods, sometimes radiating. Refractive indices (D):
$\alpha = 1.548$, $\beta = 1.550$, $\gamma = 1.558$, $\gamma - \alpha = 0.010$, all ± 0.003; intermediate values are usually shown. *In parallel polarized light, nicols crossed:* Birefringence moderate, the colors being first order; extinction inclined, reaching a maximum angle of 40°, although parallel extinguishing rods are not uncommon; elongation variable. *In convergent polarized light, nicols crossed:* a partial biaxial interference figure occasionally shown; 2 E large, and sign +.

Specific gravity = 1.520; mean n = 1.552; refractivity = 44.60.

β-glucoheptitol



$$\begin{array}{ccccccc} & & & H & & H & \\ & H & H & O & H & O & \\ H_2OHC & C & C & C & C & C & CH_2OH \\ & O & O & H & O & H & \\ & H & H & & H & & \end{array}$$

M.P. 128–129°.

Described by Philippe.[5]

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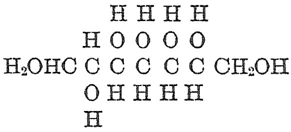
[3] Peirce, G., *J. Biol. Chem.*, 1915, xxiii, 334.
[4] Fischer, E., *Ann. Chem.*, 1892, cclxx, 64.
[5] Philippe, L. H., *Ann. chim. phys.*, 1912, xxvi, 289.

In ordinary light: seen to consist of plates and rods, the latter radiating. Refractive indices (D):
$\alpha = 1.542$, $\beta = 1.550$, $\gamma = 1.552$, $\gamma - \alpha = 0.010$, all ± 0.003; index γ is usually shown lengthwise, and β is often shown on the plates. *In parallel polarized light, nicols crossed:* Birefringence moderate, the colors being first order; extinction parallel; elongation +.

Specific gravity = 1.510; mean $n = 1.548$; refractivity = 44.61.

α-sedoheptitol
α-or β-taloheptitol



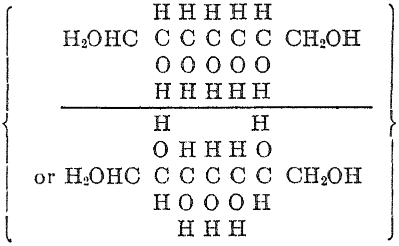
$$\begin{array}{ccccccc} & & \text{H} & \text{H} & \text{H} & \text{H} & \\ & \text{H} & \text{O} & \text{O} & \text{O} & \text{O} & \\ \text{H}_2\text{OHC} & \text{C} & \text{C} & \text{C} & \text{C} & \text{C} & \text{CH}_2\text{OH} \\ & \text{O} & \text{H} & \text{H} & \text{H} & \text{H} & \\ & \text{H} & & & & & \end{array}$$

M.P. 151°.

Described by La Forge and Hudson;[6] prepared from sedoheptose. *In ordinary light:* seen to consist of rods and needles, often bunched. Refractive indices (D):
$\alpha = 1.550$, $\beta = 1.555$, $\gamma = 1.562$, $\gamma - \alpha = 0.012$, all ± 0.003; index γ is often shown lengthwise. *In parallel polarized light, nicols crossed:* Birefringence moderate, the colors being first order; extinction parallel; elongation +.

Specific gravity = 1.520; mean $n = 1.556$; refractivity = 44.86.

β-sedoheptitol



$$\left\{\begin{array}{ccccccc} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \\ \text{H}_2\text{OHC} & \text{C} & \text{C} & \text{C} & \text{C} & \text{C} & \text{CH}_2\text{OH} \\ & \text{O} & \text{O} & \text{O} & \text{O} & \text{O} & \\ & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \\ \hline & \text{H} & & & & \text{H} & \\ & \text{O} & \text{H} & \text{H} & \text{H} & \text{O} & \\ \text{or H}_2\text{OHC} & \text{C} & \text{C} & \text{C} & \text{C} & \text{C} & \text{CH}_2\text{OH} \\ & \text{H} & \text{O} & \text{O} & \text{O} & \text{H} & \\ & & \text{H} & \text{H} & \text{H} & & \end{array}\right\}$$

M.P. 128°.

Described by La Forge and Hudson.[7]

In ordinary light: seen to consist of plates and rods, with 120° angles. Refractive indices (D) (checked by a monochromatic illuminator):
$\alpha = 1.564$, $\beta = 1.570$, $\gamma = 1.584$, $\gamma - \alpha = 0.020$, all ± 0.002; index α is often shown lengthwise. *In parallel polarized light, nicols crossed:* Birefringence moderately strong, the colors being

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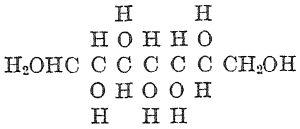
[6] La Forge, F. B., and Hudson, C. S., *J. Biol. Chem.*, 1917, xxx, 68.
[7] La Forge and Hudson,[6] p. 71.

first to second order; extinction parallel; elongation mostly −. *In convergent polarized light, nicols crossed:* a partial biaxial interference figure occasionally shown; 2 E large, and sign +.

Specific gravity = 1.590; mean n = 1.573; refractivity = 43.95.

α-guloheptitol
β-galaheptitol



$$\begin{array}{ccccccc} & & \text{H} & & & \text{H} & \\ & \text{H} & \text{O} & \text{H} & \text{H} & \text{O} & \\ \text{H}_2\text{OHC} & \text{C} & \text{C} & \text{C} & \text{C} & \text{C} & \text{CH}_2\text{OH} \\ & \text{O} & \text{H} & \text{O} & \text{O} & \text{H} & \\ & \text{H} & & \text{H} & \text{H} & & \end{array}$$

M.P. about 140°.

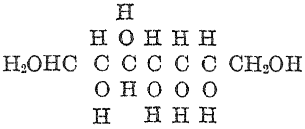
Described by Peirce[3] and by La Forge;[8] prepared from gulose.

In ordinary light: seen to consist of irregular grains. Refractive indices (D):

$\alpha = 1.554$, $\beta = 1.560$, $\gamma = 1.570$, $\gamma - \alpha = 0.016$, all ± 0.003; means of these values are usually shown. *In parallel polarized light, nicols crossed:* Birefringence moderate, the colors being first to second order; extinction apparently inclined; elongation mostly −. *In convergent polarized light, nicols crossed:* A partial biaxial interference figure frequently shown; 2E moderately large, and sign +.

Specific gravity = 1.560; mean n = 1.561; refractivity = 44.03.

β-guloheptitol
α- or β-altroheptitol



$$\begin{array}{ccccccc} & & \text{H} & & & & \\ & \text{H} & \text{O} & \text{H} & \text{H} & \text{H} & \\ \text{H}_2\text{OHC} & \text{C} & \text{C} & \text{C} & \text{C} & \text{C} & \text{CH}_2\text{OH} \\ & \text{O} & \text{H} & \text{O} & \text{O} & \text{O} & \\ & \text{H} & & \text{H} & \text{H} & \text{H} & \end{array}$$

M.P. 128°.

Described by La Forge;[9] prepared from gulose.

In ordinary light: seen to consist of pointed rods. Refractive indices (D) (checked by a monochromatic illuminator):

$\alpha = 1.565$, $\beta = 1.570$, $\gamma = 1.586$, $\gamma - \alpha = 0.021$, all ± 0.002; means of these values are usually shown. *In parallel polarized light, nicols crossed:* Birefringence moderately strong, the colors being first to second order; extinction somewhat inclined; elongation variable. *In convergent polarized light, nicols crossed:* A partial biaxial interference figure frequently shown; 2E large, and sign +.

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[8] La Forge, F. B., *J. Biol. Chem.*, 1920, xli, 254.

[9] La Forge,[8] p. 256.

TABLE I.

Properties of the Eight Heptitols Studied.

Substance	α-manno-heptitol.	β-manno-heptitol.	α-gluco-heptitol.	β-gluco-heptitol.	α-sedo-heptitol. (?)-talo-heptitol.	β-sedo-heptitol.	α-gulo-heptitol. β-gala-heptitol.	β-gulo-heptitol. (?)-altro-heptitol.
Habit	Minute needles.	Radiating needles.	Pointed rods.	Plates, rods.	Rods, needles.	Plates, rods.	Grains.	Pointed rods.
Refractive indices α	1.538	1.533	1.548	1.542	1.550	1.564	1.554	1.565
β	1.545	(?)	1.550	1.550	1.555	1.570	1.560	1.570
γ	1.549	1.545	1.558	1.552	1.562	1.584	1.570	1.586
Birefringence γ-α	0.011	0.012	0.010	0.010	0.012	0.020	0.016	0.021
Usually shown	α	γ	Means.	γ	γ	α	Means.	Means.
Birefringence	Moderate.	Moderate.	Moderate.	Moderate.	Moderate.	Strong.	Moderate.	Strong.
Order of colors	1	1	1	1	1	1–2	1–2	1–2
Extinction	Parallel.	Parallel.	40°	Parallel.	Parallel.	Parallel.	Inclined.	Inclined.
Elongation	−	+	±	+	+	−	−	±
Interference figure			Occasional.			Occasional.	Often.	Often.
Axial angle, 2E			Large.			Large.	Large.	Large.
Optical character	−	(?)	+	−	+	+	+	+
Mean n	1.544	1.539	1.552	1.548	1.556	1.573	1.561	1.574
Specific gravity	1.485	1.470	1.520	1.510	1.520	1.590	1.560	1.585
Refractivity	45.10	45.21	44.60	44.61	44.86	43.95	44.03	44.18

Specific gravity = 1.585; mean n = 1.574; refractivity = 44.18.

Discussion of Results.—It is noteworthy that the heptitols studied show in nearly every case highly distinctive optical properties, so that they could be differentiated unmistakably by this means. In the one instance of close resemblance, namely β-sedo- and β-guloheptitols, the differences between their respective α and γ values are too slight to enable them to be told apart except by the most careful work; but with a monochromatic illuminator this should be possible.

The refractivity of most of these substances comes out rather lower than the theoretical value; this must apparently be ascribed to some feature of their molecular configuration, but the exact nature of the effect is unknown.

INFLUENCE OF DIET ON THE ANTISCORBUTIC POTENCY OF MILK.*

By E. B. HART, H. STEENBOCK, and N. R. ELLIS.

(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison.*)

Plate 4.

(Received for publication, May 8, 1920.)

When Funk in 1914[1] indicated that there probably was a specific vitamine responsible for the prevention of scurvy, he expressed the belief that the diet of the milk-producing animal might be a factor in the relative antiscorbutic potency of the milks produced. This opinion was concurred in by Barnes and Hume,[2] and in a recent publication[3] we accepted this view as a possibility and a field for research. A preliminary note by Dutcher, Pierson, and Biester[4] on the same problem would indicate that they were in possession of facts tending to support this view.

There appears to be positive experimental evidence that the mammary gland does not have the power to synthesize the antineuritic vitamine and that its concentration in the milk is dependent on the supply of it in the diet.[5] However, from a recent publication by Osborne and Mendel[6] one would infer that there is no difference in the water-soluble vitamine content of milk produced under winter feeding and summer pasture feeding conditions. This is not surprising when we recall the comparative stability of this vitamine and the probability that it is not destroyed in the drying of farm crops. But their experiment in itself does not decide that there would be no variation in the water-soluble vitamine content of milk if there were a wide variation in its concentration in any two rations compared. In respect to the fat-soluble or antixerophthalmic vitamine and its relation to diet concentration and abundance in milk there are no conclusive experimental data at present. Should it be found that this vitamine is generally associated or identical with certain yellow pigments of plant tissue[7] then there is abundant evidence that the concentration of this vitamine in the milk will be related to its concentration in the ration or diet.

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* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

[1] Funk, C., Die Vitamine, Wiesbaden, 1914.

[2] Barnes, R. E., and Hume, E. M., *Biochem. J.*, 1919, xiii, 306; *Lancet*, 1919, ii, 323.

[3] Hart, E. B., Steenbock, H., and Smith, D. W., *J. Biol. Chem.*, 1919, xxxviii, 305.

[4] Dutcher, R. A., Pierson, E. M., and Biester, A., *Science*, 1919, l, 184.

[5] Andrew, V. L., *Philippine J. Sc., Series B*, 1912, vii, 67. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 33. Steenbock, H., Kent, H. E., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxv, 61.

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549.

EXPERIMENTAL.

We had excellent opportunity for studying this problem of dietary relation to the antiscorbutic vitamine concentration in milk. The University has maintained for the purpose of studying restricted ration problems a herd of eighteen cows, all of which are kept year after year on air-dried roughages and grains. No fresh green vegetable tissue of any kind has ever been fed this herd. Further, it is recognized through the results of recent investigations that the antiscorbutic properties of green plant tissue are greatly reduced in quantity even through the process of air-drying such as is customarily followed in the handling of farm crops. Such forage as finely ground alfalfa hay or corn stover[3] has been so reduced in its content of the antiscorbutic vitamine as to fail to prevent the development of scurvy in the guinea pig. Our results show that the milk from this herd was distinctly inferior in its content of the antiscorbutic vitamine to the milk derived from cows receiving a part of their ration as *summer pasture*, which consisted of fresh green material. The summer pasture milk was derived from cows of the University herd which during part of the day grazed on a timothy, blue grass, clover pasture. The amount of green plant tissue consumed by these animals was, of course, unknown.

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[7] Steenbock, H., *Science*, 1919, l, 352.

These differences in the antiscorbutic properties of milk are illustrated in Charts 1 to 8 inclusive. We used as a basal ration for the guinea pigs a mixture of heated alfalfa hay (finely ground), rolled oats, and common salt. The hay was heated in an autoclave for ½ hour at 15 pounds pressure in order to reduce further its concentration of the antiscorbutic. This ration presumably furnished a sufficiency of all the dietary factors with the exception of an ample content of the antiscorbutic vitamine. For improvement of the protein mixture, where liquid milk was omitted, five parts of casein were added. Chart A illustrates the deficiency of this ration in the antiscorbutic vitamine. The fact that nutritional failure resulted even in the presence of casein is positive proof that nutritional failure in the presence of a liquid milk addition was not due to the inclusion in the diet of too little protein of efficient supplementing character.

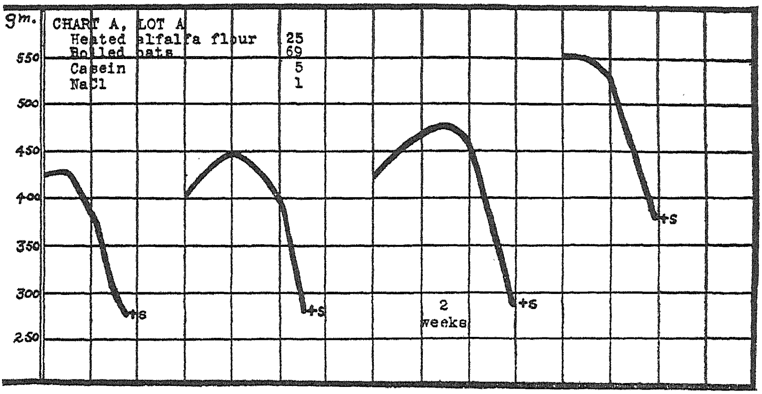

Chart A. Showing the early development of scurvy on the basal ration used in all our experiments.

Milk was given at different levels varying from 15 to 50 cc. per individual daily in the case of the summer pasture milk and from 15 to 100 cc. per individual in the case of the dry feed milks. It should be noted (Chart 1) that 15 cc. daily of summer pasture milk delayed the onset of scurvy, although not preventing its eventual occurrence in two of the animals of this group. One animal, No. 2, apparently was fully protected against scurvy by but 15 cc. of such milk. This fact must emphasize not only variations of susceptibility in individuals, but the impossibility

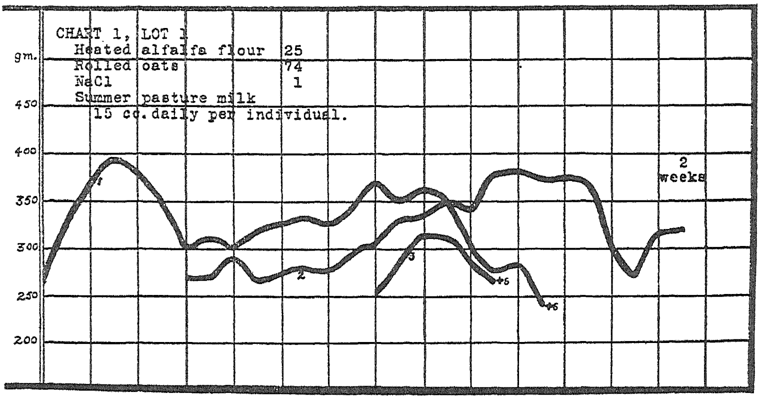

CHART 1. Showing the protection against scurvy by 15 cc. per individual daily of summer pasture milk. This amount of milk was too small for complete protection of all individuals against the disease.

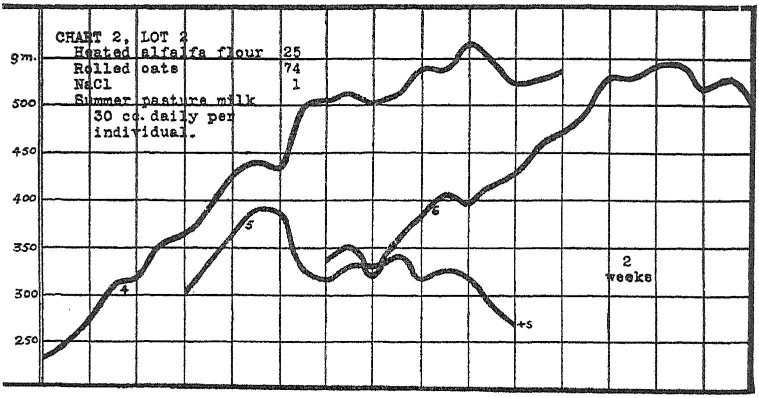

CHART 2. Showing the fuller protective action of 30 cc. per individual daily of summer pasture milk. This amount protected two animals completely, while one animal was not protected.

of fixing the minimum quantity of milk necessary for the protection of a 250 gm. guinea pig against scurvy. Where 30 cc. of summer pasture milk were allowed (Chart 2), but one animal in a group of three developed scurvy. With 50 cc. of this type of milk consumed daily per individual none of the group developed scurvy (Chart 3). One animal from this lot, No. 9, died after 9 weeks restriction to this ration, but not from scurvy; the cause of death was undetermined. On all these rations the amount of milk allowed was completely consumed.

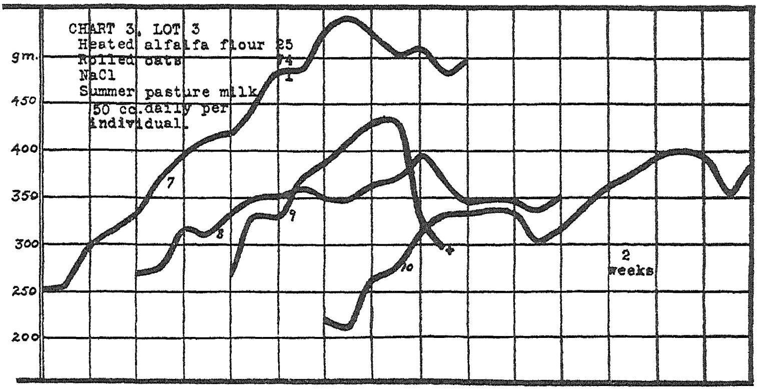

CHART 3. Showing the full protection against scurvy of 50 cc. of summer pasture milk when allowed daily per individual and used with a basal ration of rolled oats and heated alfalfa.

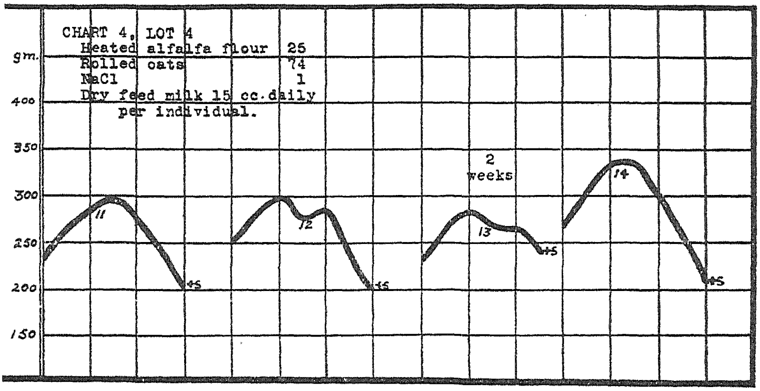

CHART 4. Showing the complete failure of 15 cc. daily per individual of dry feed milk to protect against scurvy. The basal ration was the same as in all other experiments.

Entirely different results were secured with the dry feed milk. This milk was distinctly inferior in its antiscorbutic properties to the summer pasture milk. No protection against scurvy was afforded by a daily consumption per individual of 15 cc. (Chart 4), and twice that amount, or 30 cc. of milk, was little better as a preventative, if judgment can be based on the time elapsing before the development of the disease (Chart 5). With 50 cc. of this

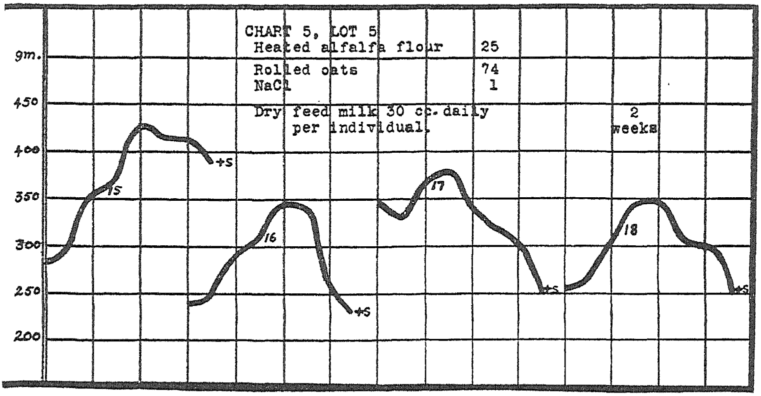

CHART 5. This chart shows that 30 cc. of dry feed milk daily per individual was insufficient as a source of the antiscorbutic vitamine.

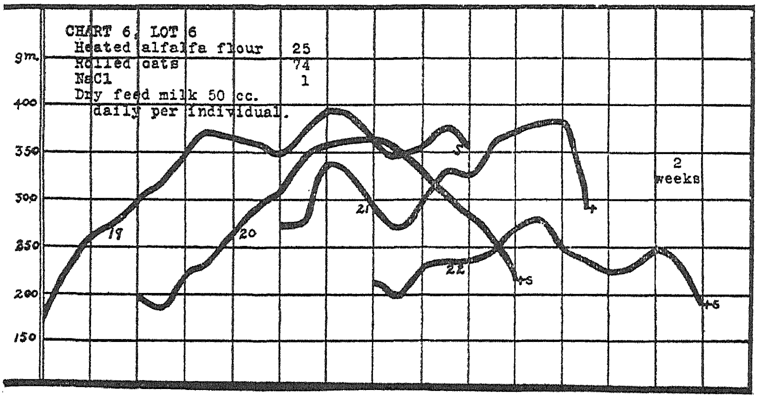

CHART 6. Not until 50 cc. of dry feed milk were allowed daily per individual was some protection against scurvy afforded. That amount only delayed the disease and did not prevent it.

milk daily per individual (Chart 6), there manifestly was some protection, but the fatality from scurvy was only delayed and not prevented. All but one of this group died with pronounced scurvy symptoms, but only after 12 to 14 weeks confinement to the ration. No. 21 of this group died after restriction to the diet for 13 weeks, but scurvy symptoms were not apparent. When the volume of milk reached 75 cc. per individual daily the protection against scurvy over a period of 18 weeks was complete (Chart 7). These particular results are instructive. They indicate that the ration used for the production of this milk was not wholly devoid of the antiscorbutic vitamine. Although dried and at least a year old the materials fed these cows still had some antiscorbutic potency. This fact probably has very great importance in its relation to the antiscorbutic needs of species other than the guinea pig or man. It demonstrates that there is a measure of this vitamine in feeds dried as these were, but that a concentrating organ such as the mammary gland and a particularly sensitive animal such as the guinea pig are necessary for a demonstration of this fact. For the first 3 to 4 weeks on an allowance of 75 cc. of milk per individual not all of it was consumed, the consumption records showing from 55 to 65 cc.

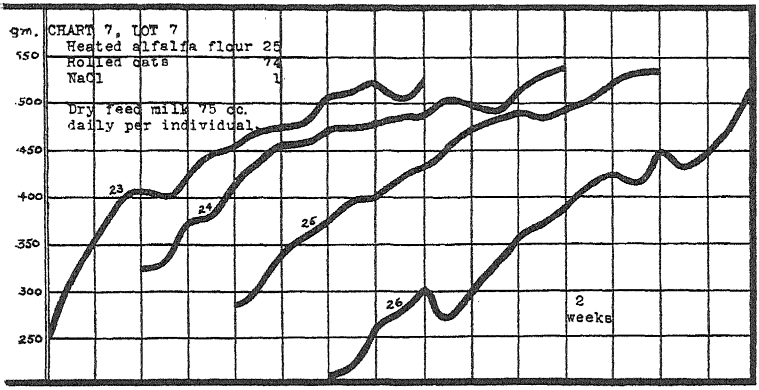

CHART 7. It required 75 cc. of dry feed milk daily per individual to protect against scurvy, while 30 to 50 cc. of summer pasture milk accomplished the same thing. This experiment shows that our dry feeds do contain some antiscorbutic, but not enough to protect a guinea pig when fed on the material directly.

per individual daily. After that time, however, the total 75 cc. were consumed.

With 100 cc. of dry feed milk daily per individual perfect protection against scurvy was afforded (Chart 8). These animals were well matured and their records are those of maintenance. For the first 10 weeks their consumption record varied from 87 to 95 cc. daily per individual. Thereafter the entire amount was consumed.

Manifestly then the diet may have a very pronounced influence upon the concentration of the antiscorbutic vitamine in the milk, being richer in this substance when the animals receive fresh green materials in the diet or ration than when the ration is made up of air-dried materials. Apparently the mammary gland has no power to synthesize this vitamine and derives it preformed from the food. These experiments make it clear that in addition to the antineuritic vitamine the concentration of the antiscorbutic vitamine in milk is dependent upon its concentration in the ration.

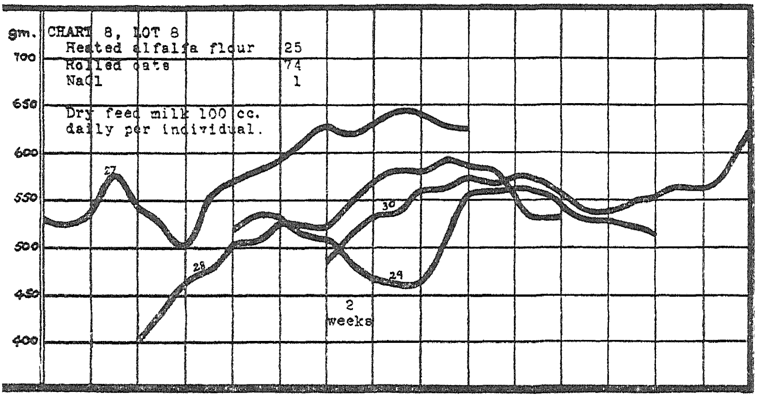

CHART 8. Showing the full protection against scurvy by 100 cc. of dry feed milk allowed daily per individual.

We next investigated the antiscorbutic properties of milk made under normal winter feeding conditions. Winter feeding of dairy cows, at least in the corn belt, generally includes corn silage in the ration. Corn silage probably varies in its antiscorbutic properties. It is possible that silage made from fresh green corn is more potent in respect to its antiscorbutic content than that made from corn killed by frost and withered and dried before it is placed in the silo. On this matter we have no data at present and will only present in this publication our experience with milk produced during the winter of 1920 from dried grains, hays, and a corn silage made from corn that had well matured and partly dried, but had not been frozen. It was put in the silo with the addition of water, a practice necessitated by its dryness. Whether processes of fermentation, involving particularly carbohydrate metabolism and acid production such as occur in silage making,[6] are destructive to the antiscorbutic vitamine is under investigation. We shall use the expression "winter-produced milk" as indicating a ration containing silage of the character described above.

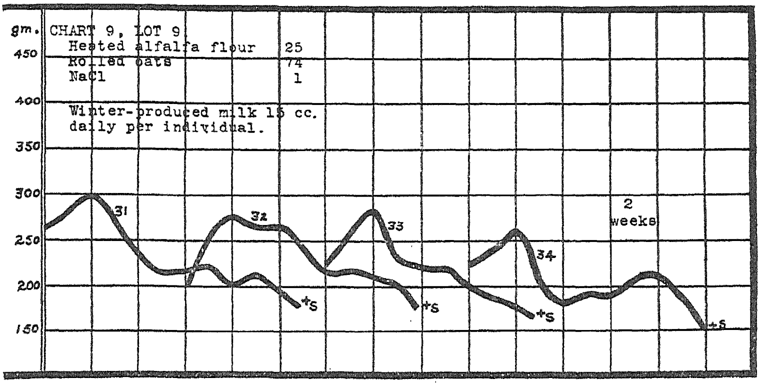

CHART 9. 15 cc. per individual daily of winter-produced milk afforded little protection against scurvy. This milk was from cows receiving a ration of dried grains and dried hays + silage, made from well matured and field-dried corn, but non-frosted.

The basal ration used for the guinea pigs was the same as in all other experiments and consisted of heated alfalfa hay, rolled oats, and common salt. The alfalfa was ground as a meal and mixed with the oats and salt in the proportion shown in the charts. With 15 cc. of winter-produced milk (Chart 9) all individuals succumbed to scurvy in 10 to 12 weeks. There was an initial gain in weight, but thereafter a gradual decline to death. These animals as a group lasted somewhat longer than the group receiving 15 cc. daily per individual of dry feed milk which would indicate either that the individuals were more resistant or that the milk was slightly richer in antiscorbutic properties. However, its potency in the antiscorbutic was in a class with the dry feed milk and not with the green pasture milk. When 30 cc. of winter-produced milk were allowed per individual daily three of the group developed scurvy early, but prolonged their existence for 9 to 10 weeks (Chart 10).

One animal in this lot, No. 38, developed scurvy symptoms as early as the other members of the group, but lived much longer. There was a slow but gradual decline in weight by this individual as the scurvy symptoms became more aggravated, although it was not dead at the end of 18 weeks.

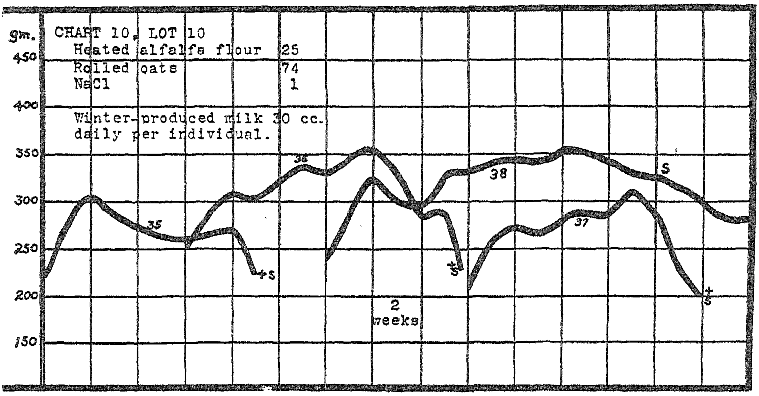

Chart 10. 30 cc. per individual daily of winter-produced milk (silage) was ineffective as an antiscorbutic when used with our basal ration of heated alfalfa flour and rolled oats.

With 50 cc. or more of this milk daily per individual the protective action was more nearly complete (Chart 11). Three of this group appeared to be protected against scurvy for a period of 15 to 16 weeks. One of the group, No. 41, developed scurvy in the 11th week and died of the disease in the 14th week. These results should be contrasted with the records shown in Charts 3 and 6, respectively. With 50 cc. of summer pasture milk allowed daily per individual none of the group developed scurvy. With 50 cc. of dry feed milk three developed scurvy and one died from some unknown cause. On 50 cc. of winter-produced milk one of the group developed scurvy. An allowance of 75 cc. daily per individual of winter-produced milk fully protected all the animals in Lot 12 against scurvy (Chart 12).

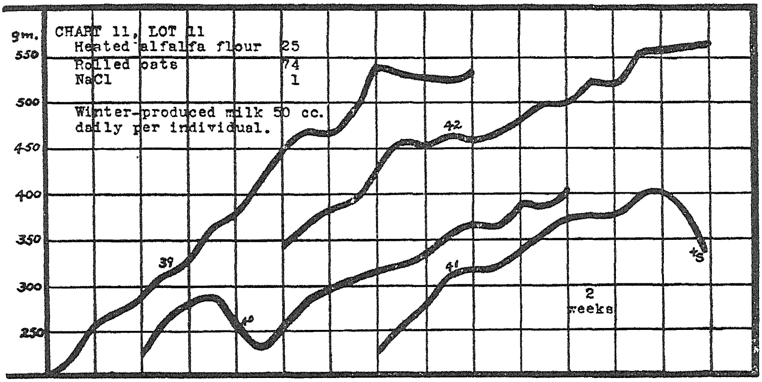

Chart 11. When 50 cc. of winter-produced milk were allowed daily per individual there was considerable protection against scurvy. Three of the group were fully protected for 16 weeks; one of the animals developed scurvy at the end of 11 weeks. Evidently this amount of milk was an insufficient source of the antiscorbutic vitamine for complete protection of all individuals.

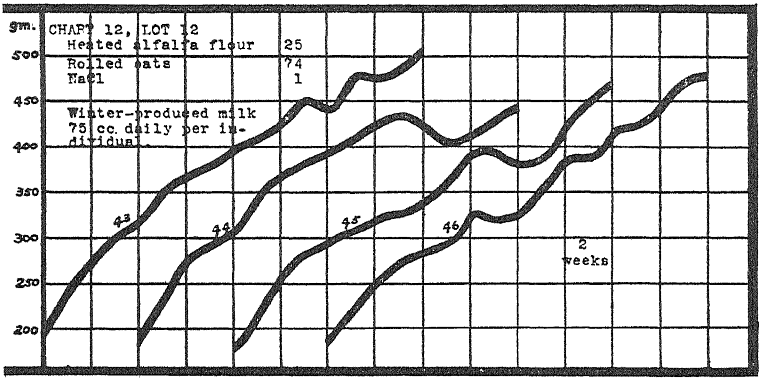

Chart 12. Showing the complete protective action against scurvy of 75 cc. daily per individual of winter-produced milk over a period of 14 weeks.

The winter feeding of dairy cows may very often include the roots of certain plants. Those most likely to be used in this country are such roots as the mangel, sugar mangel, or sugar beet. Carrots or parsnips may at times have limited use, but such roots as the rutabaga or turnip—the former reported by English investigators[8] to be comparatively rich in the antiscorbutic—are not likely to find a place in the feeding of dairy cows. This is due to the danger of producing milk tainted with the odor of sulfur oils. Our observations are limited to milk produced by a ration consisting of grains, mixed hay, a small amount of silage (10 pounds per day), and the hybrid sugar mangel (*Beta vulgaris, var.*). This root is a cross between the mangel and sugar beet. About 30 pounds of the sliced beets were fed daily per individual. Chick and Rhodes have reported that the raw beet root—presumably the sugar beet—has relatively low antiscorbutic properties; they found, by the use of 20 cc. of the juice from this root, that protection to guinea pigs against the disease was negative, while 2.5 cc. of raw Swede juice (rutabaga) prevented the development of scurvy.

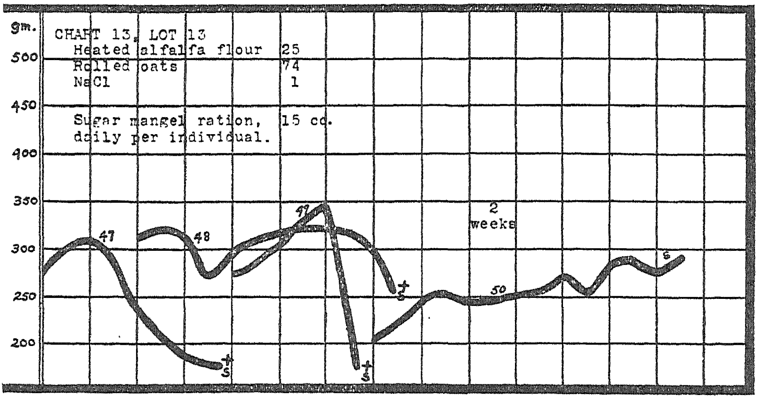

CHART 13. Showing the antiscorbutic properties of a milk produced from grain, dried hay, 10 pounds of silage, and 30 pounds of sliced sugar mangels daily. The silage was the same as used in all other experiments. 15 cc. of this milk per individual daily did not protect three of the animals in this group against scurvy. One animal was protected for 11 weeks, when initial symptoms of scurvy developed.

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[8] Chick, H., and Rhodes, M., *Lancet*, 1918, ii, 774.

The sugar mangel milk was relatively low in antiscorbutic properties. 15 cc. of this milk added to our regular basal ration afforded little protection to growing guinea pigs (Chart 13). This protection was similar to that shown by the silage-produced milk. With 30 cc. of this milk allowed daily per individual (Chart 14) the onset of scurvy symptoms was not delayed in two of the cases, Nos. 51 and 52, but Nos. 53 and 54 were protected against scurvy for 11 weeks. Apparently the concentration of the antiscorbutic vitamine in the sugar mangel milk was not of such a magnitude as to prevent in all individuals the development of scurvy on our basal ration plus 30 cc. of this milk daily.

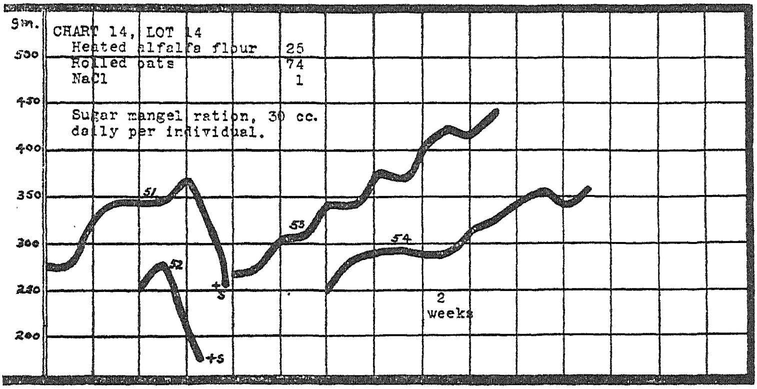

CHART 14. 30 cc. of sugar mangel milk daily per individual failed to protect two individuals in this lot. The other two animals were protected against scurvy by this quantity of milk for 11 weeks.

SUMMARY.

1. Evidence is presented showing that the concentration of the antiscorbutic vitamine in milk is dependent upon the diet.

2. Summer pasture milk is much richer in this nutritive factor than dry feed milk or winter-produced milk, involving the use in the ration of corn silage or sugar mangels.

3. 15 cc. daily of summer pasture milk imposed upon the basal ration used afforded protection against scurvy for 20 weeks to one guinea pig. However, for most individuals this quantity

of milk was insufficient for scurvy prevention. 50 cc. was a safer limit for complete protection.

4. At least 75 cc. daily per individual of dry feed milk were needed for complete protection against scurvy. The fact that milk produced from such dry feeds as used contained a measure of the antiscorbutic vitamine shows that the dry feeds themselves are not completely devoid of this nutritive factor. Their content in this vitamine is too low for protection of a guinea pig against scurvy, but the mammary gland, through a process of special selection and concentration in the milk produced, becomes an effective means of showing the presence of the antiscorbutic, especially in old dried feeds.

5. Silage made from corn well matured and partly dried before putting into the silo did not greatly enhance the concentration of the antiscorbutic factor in the milk. The same statement applies to the use of sugar mangels. Silage or sugar mangel milk was in a class with the dry feed milk and not of the order of green summer pasture milk in respect to its content of the antiscorbutic vitamine. The sugar mangel milk was a somewhat better source of this vitamine than the silage milk, but our data on this point are at present too limited to warrant an unqualified statement.

EXPLANATION OF PLATE 4.

Fig. 1. A normal guinea pig. 15 cc. daily of summer pasture milk protected against scurvy when the remainder of the ration consisted of heated alfalfa flour 25 parts, rolled oats 74 parts, and common salt 1 part. Photographed in the 12th week of restriction to this diet.

Fig. 2. A scorbutic guinea pig. 15 cc. daily of dry feed milk did not protect against scurvy when the remainder of the ration consisted of heated alfalfa flour 25 parts, rolled oats 74 parts, and common salt 1 part. Photographed after 4 weeks restriction to this diet.

Fig. 3. Showing early stages of scurvy. 15 cc. daily of winter-produced milk (silage + dry grain and dry hay) did not effectively protect against scurvy when the remainder of the ration consisted of heated alfalfa flour 25 parts, rolled oats 74 parts, and common salt 1 part. Photographed after 4 weeks restriction to the diet. The silage was made from field-dried, but non-frosted corn.

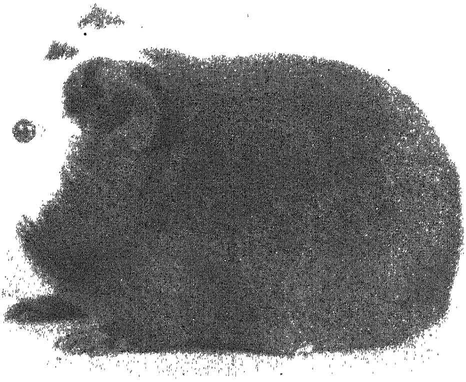

Fig. 1.

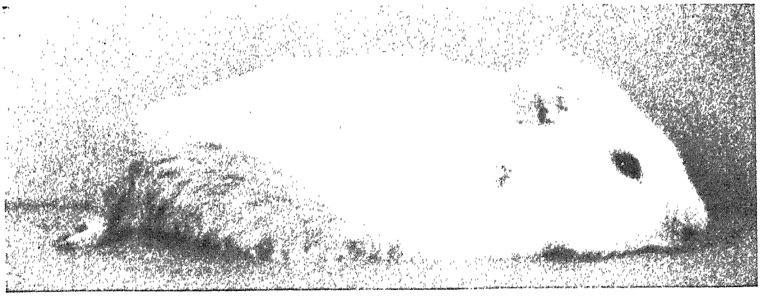

Fig. 2.

Fig. 3.

(Hart, Steenbock, and Ellis: Antiscorbutic potency of milk.)

HYDROGEN ION CHANGES IN THE MOSAIC DISEASE OF TOBACCO PLANTS AND THEIR RELATION TO CATALASE.

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(Received for publication, May 13, 1920.)

A number of determinations have been made upon the catalase[1] and oxidase[2] relations in the mosaic diseases. The author has confirmed the findings of previous authors that the catalase activity is decreased and the oxidase activity (Bunzell) increased in the mosaic areas. To account further for the abnormal growth in the infected areas and for the decrease in catalase, an attempt was made to determine the H^+ relations in the healthy and diseased tissues.

H^+ concentration determinations were made by the potentiometric method on freshly expressed juices. Tobacco leaves were collected from material of known heredity grown by Dr. Allard of the Division of Tobacco Investigations, at Arlington, Virginia. In all cases samples of 10 pounds or more of leaves were taken at the same time from healthy and diseased plants of the same variety growing in the same soil. The samples were ground in a meat chopper and expressed by hand through cheese-cloth, or a portion was taken from the middle of each leaf and treated as above. In every case each sample was made up of old and young leaves from four or more plants. It was found that tobacco leaf juice could be diluted somewhat without changing the H^+ concentration. Hence it was expected that minor differences in concentration due to the method of expression would not affect the H$^+$ concentration of the juice greatly. By using the same method for all samples the results should be comparable. Better methods are desirable but not available. The permissible dilution is not sufficiently great to allow a colorimetric method to be used for determining the H$^+$ concentration on the dark-colored juice. The concentration of buffer is low and for this reason the expressed sap is subject to considerable change of H$^+$ concentration during protein precipitation. For this reason it is necessary to work rapidly with such juices and a colorimetric method would be desirable. Freezing of the tissue was not permissible.

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[1] Chapman, G. H., *Massachusetts Agric. Exp. Station, Bull. 175,* 1917.

[2] Woods, A. F., *Science,* 1900, xi, 17. Bunzel, H. H., *U. S. Dept. Agric., Bureau Plant Industry, Bull. 277,* 1913, 7; *J. Agric. Research,* 1914, ii, 373; *Biochem. Z.,* 1913, l, 185.

In Table I are given the changes in pH at various dilutions on the same sample of juice.

TABLE I.

Dilution of juice.	pH
Undiluted.	5.753
10 : 1	5.755
5 : 1	5.788
1 : 1	5.849

A number of tests were made at different times and in most cases the mosaic plants show an H$^+$ concentration somewhat greater than healthy plants of the same variety. In some cases the mosaic and healthy plants show approximately the same acidity. In no case was the H$^+$ concentration of healthy plants greater than that of mosaic plants within the limit of error.

Catalase determinations were made in the Van Slyke apparatus for the determination of amino-acids in a constant temperature room at 30°C. 10 gm. of fresh leaf were cut up with a knife and ground in a mortar with quartz and sufficient sodium phosphate buffer mixture (C_{H^+} 2.7×10^{-8}) or $CaCO_3$ to establish the H$^+$ concentration at a desired point. All the samples were made of portions of a large number of leaves. Dilutions of 10 gm. of leaves (green weight) to 250 cc. were found to be of a convenient concentration.

In general the catalase determinations show a lesser difference in the activity of mosaic and healthy plants than those made by Chapman. This is probably for the reason that Chapman did not regulate the H+ concentration in his experiments. If the H+ concentration is not established at about C_H^+ 10^{-8}, one has the combined effect of low catalase content in the mosaic plants, lesser activity of this catalase at the greater H+ concentration of mosaic plants, and a high rate of destruction of the catalase originally present.

In Table II are given the H+ concentration and catalase activity of a number of samples. In those cases in which the mosaic plants show an H+ concentration considerably greater than the healthy plants, the catalase activity in the same lot of leaves is correspondingly low. The presence of the virus in the mosaic-diseased areas produces a condition in which abnormal metabolism and growth are associated with hydrogen ion changes and low catalase activity.

TABLE II.

Hydrogen Ion Concentration and Catalase Activity of Mosaic and Healthy Tobacco Leaves.

Healthy.			Mosaic.		
pH	$C_H^+ \times 10^{-6}$	Average O_2.	pH	$C_H^+ \times 10^{-6}$	Average O_2.
		cc.			*cc.*
5.497	7.016	17.6	5.247	8.768	17.2
5.330	8.261	10.5	5.327	8.280	6.9
5.430	7.559	13.7	5.064	9.713	7.5
5.247	8.768	13.5	5.268	8.645	13.2
5.280	8.573	13.6	5.180	9.149	11.7
5.474	7.210		5.335	8.228	
5.403	7.760		5.297	8.470	
		19.7			17.6
		16.3			11.7
		15.3			10.7
Average.					
5.380	7.878	15.0	5.245	8.750	12.1

As in the case of acidosis of animals, a great change of H+ concentration is not likely to be found for it would result in death of the tissue. The increase in H+ concentration and decrease in catalase activity in the living diseased areas can only be small if these factors are important in the life of the cells.

SUMMARY.

The H^+ concentration of juice expressed from mosaic tobacco leaves was found to be somewhat greater than that from healthy plants, the average for mosaic plants being C_{H^+} 8.750×10^{-6} and for healthy plants C_{H^+} 7.878×10^{-6}.

Catalase activity in the mosaic leaves is decreased with increasing hydrogen ion concentration.

EVIDENCE INDICATING A SYNTHESIS OF CHOLESTEROL BY INFANTS.

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(Received for publication, May 25, 1920.)

Various foodstuffs derived from plants contain plant sterols which are closely similar to cholesterol. Fairly satisfactory evidence of the availability of these plant sterols as a source of cholesterol in the animal organism has been obtained. Fraser and Gardner[1] found that phytosterols fed to rabbits on a diet of extracted bran cause an increase in the antihemolytic power of the blood serum to the same extent as when cholesterol is added to this diet. They found also[2] that when a diet of extracted bran is followed by a bran diet from which phytosterols have not been removed there results an increase in the cholesterol content of the blood. It is therefore conceivable that cholesterol, which is so prominent a structural constituent of animal tissues, may be derived altogether from cholesterol and phytosterols present in food. The complex chemical character of cholesterol, and evidence that it is conserved within the organism by being to some extent reutilized after excretion in the bile, are points in favor of this inference.

Most of the experimental evidence at hand suggests an inability of the organism to synthesize cholesterol. Ellis and Gardner[3] from analyses of eggs and of newly hatched chicks found the same quantity of cholesterol in chicks as was found to be stored in unincubated eggs. Gardner and Lander[4] found no evidence of cholesterol synthesis in chickens fed an extracted food for several weeks after hatching. Their control chickens, however, fed on an ordinary diet, did not add to their cholesterol content during the same period. Evidence of a cholesterol intake requirement, at least during this early period of the chickens' development, is not provided by the results of this experiment. Ellis and Gardner[5] state that the amount of coprosterol in the feces of dogs and cats fed a diet of lean meat or sheep brains can always be more than accounted for by the estimated quantity of cholesterol in the food. The same investigators,[6] from determinations of the coprosterol content of stools from an adult subject who received several high protein- and fat-containing diets, are inclined to regard the cholesterol intake as equivalent to the coprosterol excretion. In these "balance" experiments the coprosterol was obtained from the non-saponifiable fraction of stool extracts by crystallizing it out several times in diluted alcohol. Coprosterol forms crystals sluggishly and it is not easy to be sure that a quantitative yield has been obtained. It is permissible to doubt whether all the coprosterol present in stool samples can in this way be collected for weighing. Moreover, the cholesterol content of the diets used by Ellis and Gardner was not directly determined, but was estimated from rather scant existing data. The balance which they found in favor of cholesterol intake cannot, therefore, be regarded as more than roughly indicated.

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[1] Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc. London, Series B,* 1909, lxxxi, 230.

[2] Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc. London, Series B,* 1909–10, lxxxii, 559.

[3] Ellis, G. W., and Gardner, J. A., *Proc. Roy. Soc. London, Series B,* 1909, lxxxi, 129.

Dezani[7] has reported that white mice fed on extracted diets over a period of several weeks not only do not lose cholesterol but actually add considerably to their cholesterol content. Analyses of six controls at the beginning of the experiment gave an average cholesterol content of 0.158 gm., whereas the average amount of cholesterol found in sixteen mice after 3 weeks on various extracted diets was 0.242 gm. The author concludes from the result that growing white mice readily synthesize cholesterol. If the assumption that the extracted diets were thoroughly free of cholesterol and phytosterols is correct, it is difficult to escape this inference. It should be mentioned that none of the diets used was adequate for continued growth and maintenance of the animals. After 2 weeks they became unhealthy and began to lose weight. As evidence that healthy mice regularly synthesize cholesterol, the result of this experiment cannot be accepted without reservation. The construction of a suitable diet for this plan of experiment, *i.e.* a diet quite free of cholesterol and phytosterols, but adequate in all other respects for growth and maintenance, presents considerable difficulties and has not yet been successfully undertaken.

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[4] Gardner, J. A., and Lander, P. E., *Proc. Roy. Soc. London, Series B*, 1913–14, lxxxvii, 229.

[5] Ellis, G. W., and Gardner, J. A., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 505.

[6] Ellis, G. W., and Gardner, J. A., *Proc. Roy. Soc. London, Series B*, 1912–13, lxxxvi, 13.

[7] Dezani, S., *Arch. farm.*, 1914, xvii, 4.

In the experiments to be reported in this paper the relation of the preformed cholesterol in the food to the total cholesterol metabolism was studied in infants by comparing carefully the intake and excretion of cholesterol. The suggestion to undertake this simple balance experiment was obtained from the observation of Müller[8] that cholesterol in the case of infants, or of adults on a continued milk diet, is not changed to coprosterol in the intestine. This fact and the probability that the methods at hand for determining cholesterol in the blood could be successfully applied to stool and milk samples seemed to offer opportunity for an accurate measurement of cholesterol taken in with the food and that excreted in the feces.

It was assumed that the finding of a regularly positive balance in favor of cholesterol intake in the case of normal infants who were gaining in weight on usual diets could be taken as evidence that the cholesterol of the tissues is probably altogether derived from the preformed cholesterol in the food, whereas a regularly negative balance under the same conditions would quite definitely indicate a synthesis of cholesterol within the organism.

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[8] Müller, P., *Z. physiol. Chem.*, 1900, xxix, 129.

Method of Determining Cholesterol.

Denis and Minot[9] have quite thoroughly studied the cholesterol content of milk, using the method devised by Bloor[10] for determination of cholesterol in the blood. In our hands this simple and direct method proved to be unreliable for accurate measurement of cholesterol in stool and milk samples. The color obtained was usually found to differ so much in quality from that of the standard that only an approximate comparison was possible. In the case of milk samples so much fat was taken out by the hot chloroform as to interfere subsequently with the transparency of the reading solution. A preliminary saponification and removal of the soaps in order to bring the cholesterol in cleaner fashion into the reading solution seemed indicated. After considerable experimenting a modification of the Autenreith and Funk[11] method was devised, by means of which the cholesterol could be obtained in an entirely satisfactory condition, and without the expenditure of the time and patience required by the original method. The alteration consisted in applying Kumagawa and Suto's[12] plan of using diluted alcohol and petroleum ether as a means of separating non-saponifiable material from soaps.

The method as applied to milk was carried out as follows.

10 cc. of milk were digested in a beaker with 20 cc. of 30 per cent KOH for 2 hours on a steam bath. The digest, which then amounted in volume to about 15 cc., was transferred with small rinsings of water to a 100 cc. graduated cylinder. 25 cc. of 95 per cent alcohol and about 60 cc. of petroleum ether were then added, and the cylinder was shaken for 5 minutes. The petroleum ether, which separated quickly and sharply from the diluted alcohol, was then blown over through capillary tubing into a separatory funnel and a fresh quantity of petroleum placed in the cylinder. After shaking the cylinder again for 5 minutes the ether was transferred to the separatory funnel. The ether in the separatory funnel was then washed with about 20 cc. of water, transferred to a beaker, and evaporated on the steam bath. The dry residue was then dissolved and transferred to a small graduated cylinder by means of small portions of hot chloroform. The color was developed and the reading made in the colorimeter in the usual way. As

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[9] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1918, xxxvi, 59.

[10] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

[11] Autenreith, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

[12] Kumagawa, M., and Suto, K., *Biochem. Z.*, 1908, viii, 315.

tested by adding known amounts of cholesterol to the aqueous digest this plan of separating the cholesterol by shaking out twice with diluted alcohol and ether was found to give results which are practically theoretical, inasmuch as the small amount of cholesterol left behind cannot be measured by the colorimeter. Moreover, the formation of an emulsion which nearly always interferes with the use of the Autenreith and Funk method for milk samples rarely takes place. In determining cholesterol in the stools the plan used was to extract a large sample of dried stool with boiling alcohol and ether (3:1) for 1 hour, with a reflux condenser, and then to saponify an aliquot of the extract. The cleanliness with which the method used separates the non-saponifiable material is indicated by the fact that the non-saponifiable fraction from stools which is practically altogether cholesterol (see below) appears on the bottom of the beaker after evaporation of the petroleum ether as wide transparent plates exactly resembling the crystalline residue obtained by evaporating an ethereal solution of pure cholesterol.

A few comparisons with results obtained by the digitonin method of Windaus[13] were made. This method is not easily applicable to stool and milk samples. In the case of milk, 25 gm. of Merrell-Soule whole milk powder were first extracted with alcohol and ether. The extract was then saponified with sodium ethylate, dried with a quantity of sodium chloride, and extracted with ether in a Soxhlet.[14] After the ether had been evaporated, the residue was taken up in alcohol and the cholesterol precipitated with digitonin. A single determination carried out in this way gave a measurement of 0.78 mg. of cholesterol per gm. of dried milk. As determined by the colorimetric method the cholesterol content was 0.80 mg. per gm. The cholesterol content of two samples of dried stools measured by the digitonin method was 19.3 and 9.1 mg. per gm. as compared with 19.9 and 10.0 mg. per gm. by the colorimetric method. Taking into consideration the amount of preliminary manipulation which is necessary to prepare the samples for the digitonin method an agreement between the two methods within 10 per cent may be regarded as fairly satisfactory. A more thorough comparison of results obtained by the two methods was not possible because of lack of digitonin.

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[13] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

[14] Ritter, E., *Z. physiol. Chem.*, 1901–02, xxxiv, 430.

Subjects and Plan of Experiments.

The subjects used were four infants taken from the hospital wards several weeks after admission for correction of nutritional disturbances, and at a time when they were gaining steadily and satisfactorily in weight.

They were comfortably immobilized on a metabolism frame for a period of 3 days. The cholesterol contained in the milk taken and in the stools passed during the period was determined by the method described above.

In order to obtain a stool collection corresponding as closely as possible to the food intake, a small amount of charcoal was placed in the first and in the last feeding of the experimental period. The food given was a milk or milk modification on which the infant had gained steadily during a fore period of from 3 to 10 days. The quantity of cholesterol present in the food was not less than is contained in the modifications of milk which are usually given to normal infants at ages corresponding to those of the subjects used for these experiments. In all except one instance the infants continued to gain during the experimental period. The food was always well taken and without evidence of disturbed gastrointestinal function, except that in the instance of Subject R while receiving whole milk there was quite marked constipation. The stools from the other infants were normal in character and frequency. It must be said that the body weights of all the subjects of this experiment were much below the average which obtains for healthy infants of corresponding ages. The extent to which they fall short of this measure of a normal state of nutrition is indicated in the tables. The assumption that at the time of experiment their nutritional process was proceeding normally is based on the fact that they were gaining gradually in weight without discernible symptoms of nutritional disturbance. It must be admitted that this premise cannot be used without reservation.

Results of Experiments.

In order to test the statement of Müller that cholesterol is excreted unchanged in infants' stools, a quantity of non-saponifiable material sufficiently large for weighing was obtained from each of the stool specimens by the method described above, except that in this case a large sample of the alcohol-ether extract was taken and after saponification the non-saponifiable material shaken out with appropriately large volumes of diluted alcohol and petroleum ether. But a single extraction with petroleum ether was made as a quantitative separation was not attempted. The petroleum ether was washed several times with distilled water, then evaporated, and the weight of the residue determined. The residue was then dissolved in chloroform and its cholesterol content measured colorimetrically. By this procedure it was found that the cholesterol in the stools from all the experimental periods was approximately equivalent to the total non-saponifiable material present (Table I). Apparently neither coprosterol nor other non-saponifiable substance was present in appreciable amounts in these stools. In two instances the melting point of the non-saponifiable material was compared with that of pure cholesterol and was found to be the same (147–148°C.). The melting point for coprosterol is given as 95–96°C. This result indicates that in all probability the cholesterol content of infants' stools is equivalent to the total cholesterol excretion.

TABLE I.

Subject.	Samples of non-saponifiable material.	Cholesterol content determined colorimetrically.
	mg.	*mg.*
O'D.	10.7	11.5
S.	12.3	13.0
R.	11.6	10.8
C. 1	10.2	9.8
C. 2	14.0	13.1

The relation of cholesterol intake to cholesterol excretion is shown in Table II. It will be seen that in all the experimental periods more cholesterol is found in the stools than was contained in the food. Moreover, the discrepancy is large, the excretion of cholesterol being 1.7 to 3.4 times the intake.

TABLE II.

Subject.	Age.	Weight.	Average normal weight.	Food per day.	Gain in weight per day during experimental period.	Cholesterol in food per day.	Cholesterol in stools per day.	Ratio cholesterol excretion: cholesterol intake.
	mos.	*kg.*	*kg.*		*gm.*	*gm.*	*gm.*	
O'D.	3	4.1	5.7	360 cc. milk. 360 " barley water. 36 gm. cane sugar.	70	0.050	0.114	2.3
S.	4	5.1	6.3	540 cc. milk. 360 " water. 45 gm. cane sugar.	25	0.072	0.212	2.9
C. 1	4½	4.9	6.6	675 cc. woman's milk.	20	0.107	0.177	1.7
C. 2	5	4.9	6.8	540 cc. milk. 360 " water. 45 gm. cane sugar.	0*	0.070	0.125	1.8
R.	19	6.2	10.9	1,200 cc. whole milk.	10	0.125	0.424	3.4

* Weight stationary.

DISCUSSION.

The fact that cholesterol excretion was found to be regularly greater than cholesterol intake in the case of several infants who were gaining in weight and receiving a usual amount of cholesterol in their food can be interpreted as evidence that cholesterol is to some extent synthesized within the organism. The large discrepancy between intake and excretion lends strength to this inference. The necessary premise that the subjects of these experiments were in an altogether normal state of nutrition unfortunately cannot be warranted, as they all were at the time convalescing from more or less serious nutritional disturbances. It may be said, however, that subjects O'D and S were as apparently vigorous and healthy infants as can usually be found in hospital wards. If cholesterol metabolism is entirely dependent on an intake of preformed cholesterol in the food, it is very difficult to understand how the subjects of these experiments managed to gain in weight and present no symptoms of disturbed nutritional processes while exhibiting a large negative cholesterol balance.

The value of the results of these experiments and of any inference which can be drawn from them depends largely on the degree of accuracy with which the cholesterol content of the milk samples was measured. Milk contains about one-tenth as much cholesterol as blood and a relatively very large amount of fat. It was in our experience not possible to make an accurate colorimetric measurement of the cholesterol without first separating it from the milk fat. We believe that a closely quantitative separation is obtained by the method described. Analyses of ten samples of cow's milk by this method gave an average result of 13.0 mg. per 100 cc., the figures ranging from 9.8 to 15.5 mg. per 100 cc. Denis and Minot using Bloor's method found an average value of 14.5 mg. per 100 cc. In the fifteen samples analyzed the cholesterol content was between 10.5 and 17.6 mg. per 100 cc. Apparently Bloor's direct method applied to milk will usually give a value for cholesterol content which is 10 to 30 per cent higher than would be obtained by the saponification method used in these experiments. The discrepancy found between the intake and excretion of cholesterol by these infants was, however, so large that, even if it is assumed that Bloor's method measures the cholesterol content correctly and the method used is debited with a minus error of 30 per cent, the fact of a large negative balance would not be altered.

SUMMARY AND CONCLUSIONS.

Analyses of the non-saponifiable fraction of infants' stools confirms the observation of Müller that cholesterol is excreted in infants' stools without undergoing to appreciable extent change to coprosterol.

Making use of this fact we determined the cholesterol intake and excretion of four infants over periods of 3 days. The subjects used were gaining in weight and were receiving a usual amount of cholesterol in their food. In all the experimental periods a much larger excretion than intake was found, the quantity of cholesterol in the stools being 1.7 to 3.4 times that which was present in the food. This result is interpreted as indicating a synthesis of cholesterol within the organism.

A method for determining cholesterol in milk and stool samples is described.

DEGREE OF ALKALINITY NECESSARY FOR THE PHLOROGLUCIN TEST FOR FORMALDEHYDE.

By PAUL J. HANZLIK.

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(Received for publication, May 25, 1920.)

The successful use of phloroglucin as a reagent for the detection of free formaldehyde depends on the recognition of two important factors; namely, purity of the reagent and degree of alkalinity. As to purity, the white crystalline variety (Merck's reagent) of phloroglucin only should be used and not the brown amorphous variety, which greatly modifies the characteristic red color with formaldehyde.

The degree of alkalinity necessary for a positive reaction with formaldehyde will be appreciated from the following observations which were made with ordinary buffer mixtures of primary and secondary phosphates, sodium bicarbonate, sodium carbonate, and sodium hydroxide of known hydrogen ion concentrations. 0.5 cc. of 1 per cent phloroglucin in alcohol was added to 10 cc. of the solutions representing the different degrees of alkalinity and containing 1:10,000 parts of absolute formaldehyde in one set of experiments and 1:100 parts of formaldehyde in another set. The results with the two different concentrations of formaldehyde were identical and are summarized in Table I.

From this it is seen that the degree of alkalinity necessary for a minimal positive formaldehyde reaction is rather high, namely pH 12.13, or the equivalent of the alkalinity of about 0.01 N solution of sodium hydroxide, or about 0.04 per cent. An optimal positive reaction requires 0.1 N hydroxide or carbonate (pH of about 13.0). The reaction cannot occur in solutions of bicarbonate since this salt cannot provide a high enough degree of alkalinity. Ammonia water and very weak solutions of sodium hydroxide also are inadequate. Lack of attention to this detail, *i.e.* the proper degree of akalinity, probably explains the unsuccessful use of the reagent in studies on formaldehyde liberation from hexamethylenetetramine by some investigators. The practice of using the phloroglucin in alcoholic solution followed by the haphazard addition of alkali is unsatisfactory because under these conditions the proper adjustment of the degree of alkalinity may be easily overlooked. Mere bluing of red litmus paper may not be sufficient. More reliable results are obtained by the use of a reagent, consisting of 1 per cent phloroglucin in 10 to 20 per cent sodium hydroxide. This keeps almost indefinitely and insures the required degree of alkalinity for all ordinary purposes, including the application of the reagent for the detection of formaldehyde in urines and other body fluids, except bile and blood, containing hexamethylenetetramine.

TABLE I.

Solution used.	Degree of alkalinity.	Formaldehyde reaction.
	pH	
0.1 N $NaHCO_3$.	About 8.2.	Negative (yellow color).
Phosphate mixture.	8.4	" " "
0.001 N NaOH.	12.12	" (yellowish color).
0.01 N NaOH.	12.13	Positive (reddish).
0.1 N NaOH.	13.07	" (deep red).
0.1 N Na_2CO_3.	About 13.00.	" " "
1 N NaOH.	14.05	" " "

Trendelenburg[1] makes use of a reagent containing 1 per cent phloroglucin in 33 per cent sodium hydroxide. The violet color which the reagent assumes if made with weaker alkali, according to him, does not occur under these conditions. After several years experience with the reagent containing 10 to 20 per cent alkali, I have found that the violet color, which develops while the phloroglucin is undergoing solution in the hydroxide, disappears on standing, leaving the reagent almost colorless, or at most yellowish. Furthermore, the violet color does not at all interfere with the development of the positive formaldehyde reaction even in extremely weak and quantitative solutions, since this depends on a brilliant red color in high and orange reddish in weak concentrations.

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[1] Trendelenburg, P., *Biochem. Z.*, 1919, xcv, 146.

In fact the gradations between the colors obtained with different concentrations of formaldehyde are fine enough for quantitative purposes. Such a method with the use of an artificial standard has been previously described by Collins and Hanzlik.[2] The same method can be used for the quantitative estimation of hexamethylentetramine.

CONCLUSIONS.

The phloroglucin test for free formaldehyde requires a rather high degree of alkalinity, *i.e.* pH 12.13, or the equivalent of the alkalinity of a 0.01 N sodium hydroxide solution, or about 0.04 per cent, for a minimal positive reaction, and 0.1 N hydroxide or carbonate for an optimal reaction (pH 13.0). The neglect of this important factor, and also the use of impure phloroglucin, may explain the unsuccessful use of the reagent by some. Used properly this is probably the most sensitive reagent for free formaldehyde known.

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[2] Collins, R. J., and Hanzlik, P. J., *J. Biol. Chem.*, 1916, xxv, 231.

EXPERIMENTS ON CARBOHYDRATE METABOLISM AND DIABETES.

I. INTRAVENOUS GLUCOSE TOLERANCE OF DOGS.

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The present paper forms one of a series concerning the internal pancreatic function. Experiments dealing with quantitative relations between this function and the body mass and metabolism have already been in part presented elsewhere.[1] The series in this *Journal* will concern the nature of the pancreatic function and its rôle in metabolism. This series is fragmentary. The study was begun in the early period of the diabetic work at The Rockefeller Institute, as an attempt either to establish or disprove certain conceptions of normal and diabetic metabolism which had grown out of the previous investigation at the Harvard Medical School. Events compelled dropping this undertaking, and only a few experiments were worked in subsequently as time permitted. The portion completed may serve to clear the ground a little and reveal the scope and purpose of the original plan.

The present paper deals with the assimilation of intravenously injected glucose by dogs. Merck's anhydrous glucose was used throughout, and the concentration of solutions determined by simple weighing. Analyses for sugar in blood or plasma were performed by the original Lewis-Benedict[2] method, and those for urinary sugar by titration with Benedict's copper solution. Except when otherwise stated, the dogs were on a diet of bread and soup *ad libitum,* and experiments were begun 20 to 24 hours after the last feeding. The method of injection requires special mention.

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[1] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, Experimental studies on diabetes, Series I, 363, 381, 555, 575, 587; xxxii, Series II (in press).

[2] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

Interrupted injections were used, essentially according to the method of Blumenthal,[3] for two reasons; first, the experiments were mostly performed before Woodyatt had devised his excellent apparatus for uniform continuous infusions; second, one important purpose of the test was the comparison of the normal assimilation with that attained during exercise, and an interrupted method seemed indispensable for this purpose. Obviously, when short injections are given 15 or 20 minutes apart, there is a sudden flood of hyperglycemia, followed by a gradual decline to the time of the next injection. The excess passes rapidly into the tissues, and there is a certain basis for Blumenthal's conception of a "saturation limit" of the organism, in that it will hold a certain quantity of sugar like a sponge, and with identical dosage the assimilation is practically the same whether the injections are given continuously or at short intervals. It is true that Wilder and Sansum[4] criticized the irregularities of tolerance found in rabbits by different workers with the Blumenthal method, but there is no evidence that these irregularities were due to anything else than the failure of the earlier workers to take the precautions against fright, pain, and shock concerning which Woodyatt and his collaborators were so careful, and Woodyatt[5] recognizes that the results with his method and the Blumenthal method are essentially the same. At any rate, the plan of delivering fixed doses of glucose into a vein and allowing fixed intervals for its disposal is one valid method of testing the assimilative power. A few experiments with continuous infusion were also performed as controls.

The detailed procedure was as follows. The dogs used were sturdy, phlegmatic animals, thoroughly accustomed to the laboratory and to staying on the table without jumping down, and with all fear or nervousness lost through previous experience. The dietary tolerance of the diabetic ones was accurately known from long observations. An hour or so before the experiment, an area of the neck was shaved, if necessary, and a short portion of an external jugular vein exposed, with local anesthesia. The essential point is to clean a tiny portion of vein wall of every trace of fascia. The skin falls together so that the uncovered area is kept naturally moist. The dog is not tied or confined at any stage of the experiment. At the time for beginning, an assistant raises the dog's head. The operator takes a Luer syringe containing a few crystals of potassium oxalate and equipped with a needle, of No. 21 or 22 size and $\frac{5}{8}$ inch length. He draws blood into the syringe, then detaches the needle without removing it from the vein, and immediately attaches another syringe previously filled with the solution at proper temperature and makes the injection at a convenient rate, requiring perhaps 2 to 4 minutes according to the dose. There is no bleeding when the needle is withdrawn. The dog sits or lies quietly without restraint, generally dozing, till the time of the next injection. The needle is then inserted through the same hole in the vein wall as before, the blood sample drawn, and the injection made as before. The same hole in the vein is generally used throughout the whole experiment. The blood sugar values thus obtained are a minimum, because taken at the end of the interval. A few analyses have been made immediately after injection. In some experiments blood samples were taken only hourly. The method serves satisfactorily for comparisons of assimilation, but affords no exact reckoning of relations between the sugar levels in blood and urine.

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[3] Blumenthal, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 329.

[4] Wilder, R. M., and Sansum, W. D., *Arch. Int. Med.*, 1917, xix, 311–334.

[5] Woodyatt, R. T., *The Harvey Lectures*, 1915–16, xi, 326.

Continuous injections were given through a small cannula tied into an external saphenous vein, while blood samples were taken from the external jugular. Here also the dogs were merely watched and not confined; they mostly dozed without noticing the cannula, but were free to rise and stretch as suited their comfort. The glucose solution was merely run in from a burette fixed at a suitable height. This generally held the quantity to be given in 15 minutes, and it was one person's duty to keep the flow uniform and in particular to make sure that one-third of the quantity was delivered every 5 minutes.

With both kinds of injections, the dogs were catheterized hourly or sometimes at shorter intervals. Permanent catheterization was not used. The glucose solutions were made up in distilled water except when saline is specified.

REMARKS AND CONCLUSIONS.

1. Agreement of Results.—As shown in Table XXXII, the results in normal dogs agree satisfactorily both among themselves and with Woodyatt's findings. Giving 1.8 to 2 gm. per kilo per hour, Sansum and Woodyatt[6] found ordinarily about 2 per cent of the dose excreted, with variations between individual animals reaching as high as 10 per cent of the dose in one animal. A similar close agreement is seen here between the dogs receiving 1.5 gm. per kilo per hour. Also in harmony with Woodyatt, the dosage of 1 gm. per kilo per hour is found to be slightly above the tolerance, with the single exception shown in Table IV, Period A. Variations in the concentration have, as shown by Woodyatt, no perceptible effect upon the assimilation. Even the different modes of administration, whether continuously or in four, three, or even two injections per hour, are apparently of minor importance, though this is not certain, and it is desirable that comparisons should be based upon the same method, as has been done in this series.

2. Demonstration of Alterations of Tolerance.—(A) A reduction of tolerance, as established by Wilder and Sansum[4] in human patients, is demonstrable in a broad sense in diabetic animals. Tables XXXII and XXXIII show that in general the excretion of sugar in partially depancreatized dogs is greater than that in normal dogs, and that this excretion increases with the severity of the diabetes. Also Tables XXIX, XXX, and XXXI show increase of the sugar excretion with aggravation of the diabetes in the same animal, and the latter two were thus used[7] to demonstrate the downward progress of diabetes on excessive diets of either carbohydrate or protein even in absence of glycosuria.

(B) This general rule is subject to very marked exceptions. A mildly diabetic animal (Table XX) remained free from glycosuria with dosage of 1 gm. of glucose per kilo per hour, the tolerance thus apparently surpassing that of normal animals. The difference between Periods A and E in Table XVII evidently indicates aggravation of the diabetes during the experimental period, but even at the close the diabetes was still very mild, so that the dog was able to live on bread without glycosuria. Yet the excretion of 37 per cent of the dose by this animal was higher than that of any of the other dogs of the series, some of which were so severely diabetic that they were kept alive only on low protein-fat diets and would have gone into a hopeless condition if this tolerance had been overstepped for only a few days. The dog in Table XXVIII had one of the severest grades of diabetes in the series, which was thoroughly controlled by diet and reduction of weight. It was proper that the test should show the benefit of such thoroughness, but not that the assimilation should appear superior to that of the two non-diabetic dogs (Tables XV and XVI) and of nearly all the mildly diabetic ones. Other comparisons between diabetic dogs in Table XXXIII show that the sugar excretion is seldom an accurate measure of the degree of diabetes as known from the food tolerance and general clinical condition. The same applies to the gradations of assimilation in conditions short of diabetes. Thus the excretion is practically identical in Tables II and XV, though one animal was normal and the other was depancreatized to an extent involving a very great lowering of tolerance as demonstrable by alimentary and subcutaneous tests.[1] The animal in Table XVI, which was very close to diabetes, excreted a bare trifle more glucose than the normal dog in Table VI and actually less than that in Table VIII. Tests of assimilation based upon the rate of excretion in comparison with the rate of intravenous injection of glucose are therefore subject to wide errors.

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[6] Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1917, xxx, 155–173.

[7] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 567, 569.

(C) Determinations of the concentration of sugar in the blood are necessary for the correct interpretation of such experiments.

(*a*) This is the only means by which the important factor of the renal function can be judged. The latter is the outstanding cause of the apparent irregularities of tolerance in these experiments. The normal dog in Table IV showed no more than a doubtful reaction in the urine when the blood sugar in Period A was found as high as 0.173 per cent. It is self-evident that these analyses taken just before injections represent minimal values. This fact has occasionally been demonstrated by taking samples from the opposite jugular or a femoral vein as quickly as possible after finishing an injection; for example, a value of 0.457 per cent was thus found in Table III, and one of 0.770 per cent in the diabetic dog in Table XXX, and 0.294 per cent about 2 minutes after an injection in Table XIX, Period A. This flood of sugar rapidly passes from the blood into the tissues, as already mentioned. It is obvious that the normal dog in Table IV had an exceptionally high renal threshold for sugar in Period A, especially as the temporary excess of sugar was necessarily greater with only two injections per hour; also in Period B the sugar elimination was relatively small compared with the hyperglycemia. This renal impermeability will readily explain the exceptionally high tolerance. The mildly diabetic dog in Table XVII had an exceptionally active excretory function for sugar. After discontinuance of injections in Period A, this dog had glycosuria with plasma sugar concentrations from 0.084 to 0.116 per cent; and in this and the other experiments the quantity of sugar excreted was high in comparison with the hyperglycemia. With increasing severity of diabetes, the dogs were kept on protein-fat diets, and accordingly showed uniformly high renal thresholds.[8] Thus, after discontinuance of injections, in Table XXVII there was a negative sugar reaction in the final urine sample with the plasma sugar 0.208 to 0.226 per cent. In Table XXX, Period A was performed while the dog was aglycosuric on bread diet, and there was faint glycosuria at the close with plasma sugar about 0.130 per cent; Period B was performed (November 19) after a change to protein-fat diet had become necessary on October 10, and at the close there was faint glycosuria while the plasma sugar was falling from 0.322 to 0.156 per cent, and none while it was falling from 0.156 to 0.147 per cent; Period C was performed after continuance of protein-fat diet to the following February, and in the final period there was faint glycosuria while the plasma sugar was falling from 0.294 to 0.170 per cent. Dog D4-69 (Table XXXI) was an animal with one kidney, which at different times showed plasma sugar of 0.256 to 0.264 per cent without glycosuria. Also in these animals the quantitative sugar excretion was generally low in comparison with the hyperglycemia. The writer previously[9] suggested fallacies in the apparent exactness of intravenous tolerance tests, particularly the abnormal prominence of the factor of renal permeability in them. Woodyatt and his collaborators have adopted the rate of utilization as a standard, and have striven to avoid the errors of Blumenthal's "saturation limit," but their publications contain no records of tests upon nephritics or others with elevated renal thresholds.

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[8] Data will be presented in Paper II of this series. Allen, F. M., *J. Biol. Chem.*, 1920, xliii (in press).

[9] Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, 53, 54.

(*b*) The lowered tolerance of the animals which were diabetic or near to diabetes was invariably manifest in their greater hyperglycemia. Three types of plasma sugar curves may be distinguished in the longer injections. First, the normal curve seems to be a plateau which is maintained with only accidental irregularities for as long as 10 hours, as in Table III. A greater number of experiments would have been desirable to establish this as a general rule and exclude exceptions, such as a possible falling tendency of the curve indicating improved utilization due to some reaction of the pancreas or general organism; but Woodyatt's experience that glycosuria rises for the first few hours and then maintains a constant level is opposed to any such reaction of improved utilization. Second, it was noticed[1] that at certain stages of partial pancreatectomy or diabetes there is some kind of reaction which increases the apparent tolerance for glucose given by stomach or subcutaneously, and the microscopic studies also prove that the pancreatic islands can be driven to overfunction by carbohydrate excess. Occasional animals in the proper stage of mild diabetes showed such a reaction in the intravenous tests; *e.g.*, in Table XX a plasma sugar plateau of about 0.2 per cent was maintained over 2 hours, and then fell to 0.128 and 0.112 per cent; and in the longer experiment of Table XXX, Period A, this was more marked, for the plasma sugar which rose as high as 0.715 per cent at the close of the 2nd hour declined gradually to 0.125 per cent at the end of the 8th hour. Sometimes the reaction seems to break under longer strain, so that a transitory fall of plasma sugar concentration is followed by a secondary rise, as in Table XV, Period A. These declines were not explainable by changes of sugar excretion. Third, this power of the pancreas remnant to react to carbohydrate excess is lost in the more severe stages of diabetes. The progress of this loss is seen in Periods B and C of Table XXX; and the other animals with the more severe grades of diabetes show merely an excessive hyperglycemia with no tendency to decline.

(*c*) The combined consideration of the glycosuria and the blood sugar concentration makes the intravenous method sufficiently accurate for practical tests of tolerance, and it holds a position of special usefulness in excluding irregularities of absorption, notably in such conditions as thyroid and pituitary deficiency. Outside of such special conditions, other factors seem to predominate over any possible irregularities of absorption, and it is doubtful if the intravenous method can show the finer gradations of tolerance such as are revealed by the alimentary or subcutaneous administration of glucose. Any lowering of assimilation demonstrable by the intravenous method in this series was fully obvious in feeding tests. On the other hand, Table XVI shows a dubious outcome of the intravenous test in an animal which was very close to diabetes; and in Table XV the test is still more doubtful in an animal possessing only $\frac{1}{3}$ to $\frac{1}{4}$ of the pancreas, in which the results of alimentary or subcutaneous tests are usually plain. It is unfortunate that further tests could not be performed upon animals possessing larger fractions of pancreas. The reasons for the differences between the different modes of administration will receive fuller discussion at the close of this series of papers.

3. Water Balance.—*A. Body temperature.*—A slight elevation of rectal temperature was the rule in the experiments, as shown in Table IV and incidental observations in Tables XIII, XV, and XVII. The dogs were comfortable and refused offered water, with the single exception in Table XX. Any serious desiccation is therefore excluded.

B. Hydremia.—Hemoglobin estimations by the Fleischl-Miescher method were performed in three normal (Tables II, IV, and XIV) and seven partially depancreatized dogs (Tables XV to XIX, XXVII, and XXVIII). Some of the dogs were anemic from cage life and repeated bleeding experiments, but not to any degree apparently invalidating the experimental results. A fall in hemoglobin during the injection period was the rule, sometimes with a return to the original level at the close and sometimes not. One exception was seen in the rise of hemoglobin in the diabetic dog in Table XXVIII, Period B. There was no uniform relation with the plasma sugar level and no special difference between diabetic and non-diabetic animals. This behavior of the hemoglobin is to be expected when the blood is diluted by injection of an aqueous solution, and the determinations serve chiefly to prove that the variations in plasma sugar were not explainable by dilution or concentration of the blood. The plasma bicarbonate concentration fell during the injection period in Table XV, Period C, though the fat absorption is a possible factor here; but in Table IV, Period A, it actually rose.

C. Diuresis.—The urine was regularly less than the quantity of solution injected, and there was more or less antidiuretic action of glucose, in the sense of retention of water in the blood or in the body, in both normal and diabetic animals. On the other hand, in one diabetic animal (Table XXVII) a comparison was made between 0.9 per cent saline and 5 per cent glucose in 0.9 per cent saline. Here the hydremia was less and the urine volume greater with the glucose solution, so that in this sense glucose was a diuretic.

In relation to all the above observations, notice should be taken of one fact to avoid confusion in interpretation. Even in severe diabetes, if the tolerance has been conserved so that the greater part of a single dose of sugar is assimilated in a manner approaching the normal, the behavior in other particulars, such as diuresis, should likewise resemble the normal. The similarity of behavior of the diabetic and non-diabetic animals in this series conforms to this expectation.

4. Influence of Protein and Fat Feeding.—In connection with the question whether the endocrine pancreatic function is directly and primarily concerned with carbohydrate metabolism alone or also with protein and fat metabolism, it was desired to perform comparative assimilation tests in fasting and fed animals. Later papers in this *Journal* will corroborate existing evidence that fat feeding causes no immediate glycosuria or hyperglycemia; but in the more severe stages of diabetes protein feeding causes prompt and marked elevations of blood sugar. The greatest importance would therefore be attached to any positive results in normal and mildly diabetic animals, in which protein alone causes no hyperglycemia. In a previous experience[10] feeding tests had shown no important differences of tolerance for starchy foods alone or with protein, but a control with intravenous glucose tests was desired

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[10] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 401.

sible alterations of absorption. The normal dog
s given identical intravenous glucose injections,
g and Period B 5 hours after feeding 500 gm. of
e glycosuria was practically identical in the two
the hyperglycemia was slightly greater in the fast-
especially in the early part of the test (probably
tion of "hunger glycosuria"). The partially de-
on-diabetic dog in Table XV was similarly tested
otein, and after fat feeding. Here also the plasma
its highest concentration of 0.208 per cent at the
hour in the fasting period. Otherwise the plasma
d not differ appreciably and the glycosuria was
ntical. The mildly diabetic dog in Table XVII
in Period A fasting; then Period B with lard feed-
tinctly higher hyperglycemia and glycosuria; then
control feeding of clay (swallowed readily in the
olded balls placed in the throat) showed an equal
but less glycosuria; then Period D with lard feed-
higher hyperglycemia than before; finally Period
a feeding showed the highest hyperglycemia of all.
prevented a final fasting test, but the record prob-
merely a gradual aggravation of the diabetes and
arded as proof of an influence of fat or protein feed-
sugar assimilation. It may be mentioned inciden-
gestion of anything seems sometimes to raise the
l sugar, as seen in Periods B, C, and E, and the ef-
rely psychic as shown by the negative effect of tan-
eriod E. The mildly diabetic dog in Table XIX
ore than accidental variations in hyperglycemia and
hen tested fasting or after protein or fat feeding.
question was raised by the severely diabetic dog in
II, which was known from other tests[11] to be sub-
ed hyperglycemia from protein feeding alone. Here
s in glycosuria were trivial, and the hyperglycemia
in the fasting test (Period A), being slightly lower after
markedly lower after fat feeding. The vomiting of
ung by this dog after the close of the injections, of
log in Table XVII, Period D, and of clear water in

M., *Am. J. Med. Sc.*, 1917, cliii, 363, 365.

the fasting period of Table XIV, raises the question whether intravenous glucose injections interfere with alimentary absorption. There was marked milkiness of the plasma in Table XV, Period C, but little or none in the other fat-feeding experiments. The experiments at least afford no evidence of a direct participation of the pancreatic hormone in protein or fat metabolism, but their decisiveness is open to question.

TABLE I.

Dog F6-18.

Weight 11 kg. Intravenous glucose injections, 1 gm. per kg. per hr. in 10 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Dec. 20	3.25 p.m.	27	0	0.086	3.50 p.m. started injections.
	4.45 "	18	0.56	0.109	
	5.45 "	8	0.77	0.118	5.35 p.m. finished injections.
	7.10 "	56	0	0.112	
	8.10 "	40	0	0.109	Total solution injected.... 220 cc. Total glucose injected.... 22 gm. Total urine excreted ..... 122 cc. Total glucose excreted.... 0.16 gm. Per cent of dose........ 0.73 per cent.

TABLE II.

Dog C3-93.

Weight 15.5 kg. Normal. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.				Plasma sugar.		Hemoglobin.		Remarks.
		Volume.		Glucose.						
		A	B	A	B	A	B	A	B	
1916		*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
May 1	9.00 a.m.						0.117		105	Fed 500 gm. lung.
Period A.	11.00 "						0.114		98	
May 31	2.00 p.m.			0	0	0.133	0.111	105	100	Started injections.
Period B.	2.20 "	5	19	2.08	0.29	0.153	0.112	92	87	A=Fasting.
	2.40 "	8	18	1.64	0.82	0.132	0.116	85		B=After feeding 500 gm. lung.
	3.00 "	8	20	1.05	0.80	0.095	0.123	89		
	3.20 "	4	13	1.72	0.97	0.119	0.084	98	100	
	3.40 "	5	45	1.52	Faint.	0.103	0.080	104	100	
	4.00 "	6	70	1.39	"	0.098	0.099	106	102	
	4.20 "	13	12	0.80	0.54	0.092	0.128	110	98	
	4.40 "		31		0.75	0.143	0.114	111	100	Finished injections.
	5.00 "	4	15	0.74	0.68	0.161	0.118	105	100	
	5.30 "	18		0		0.105	0.093	104	95	
	7.00 "	58	190	0	Faint.	0.104	0.095	105		

Periods A and B.	
Total solution injected	155 cc.
" glucose "	31 gm.
Period A.	
Total urine excreted	129 cc.
" glucose "	0.67 gm.
Per cent of dose	2.16 per cent.

Period B.	
Total urine excreted	433 cc.
" glucose "	0.89 gm.
Per cent of dose	2.87 per cent.

TABLE III.

Dog F6-03.

Weight 20 kg. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Nov. 19	10.45 a.m.	70	0	0.119	11.10 a.m. started injections.
	12.05 p.m.	12	2.06	0.149	
	1.05 “	4	2.12	0.185	
	2.05 “	21	2.38	0.172	Plasma sugar immediately following injection 0.457 per cent.
	3.05 “	15	0.76	0.147	
	4.05 “	124	0.35	0.147	
	5.05 “	65	0.52	0.159	
	6.05 “	77	0.47	0.200	
	7.05 “	92	0.38	0.175	
	8.05 “	86	0.41	0.164	
	9.05 “	78	0.23	0.196	8.55 p.m. finished injections.
	10.00 “	35	Faint.	0.113	
	11.00 “	39	0	0.110	Total solution injected....1,000 cc. Total glucose injected.... 200 gm. Total urine excreted...... 648 cc. Total glucose excreted.... 2.95 gm. Per cent of dose........ 1.48 per cent.

TABLE IV.

Dog C3-47.

Weight 15 kg. Normal. Comparative intravenous glucose injections of 1 and 1.5 gm. per kg. per hr. (2 injections per hr.).

Date.	Time.	Urine.				Plasma sugar.		CO_2		Hemoglobin.		Temperature.		Remarks.
		Volume.		Glucose.		A	B	A	B	A	B	A	B	
		A	B	A	B									
1916		*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*per cent*	*°F.*	*°F.*	
Jan. 21	1.30 p.m.	150	171	0	0	0.120	0.133	50		121	100	101.8	101.4	1.40 p.m. started injections.
Period	2.00 "	25	40	(?)	1.56	0.173	0.232	55.7		82	80	102	101.6	
A.	2.30 "	69	145	0	0.32	0.136	0.356	54.8		81	83	102	101.6	
Jan. 27 Period	3.00 "	119	245	0	Very faint.	0.154	0.130*	55.7		79	103*	102.2	102.1	3.06 p.m. finished injections.
B.	3.30 "	176	50	0	0.37	0.125	0.196	59.5		84	88	102	102	
	3.40 "	110	95	0	0	0.114	0.128	59.5		84	87	101.6	101.8	

A. 1.0 gm. per kg. per hr. in 5 per cent solution.
Total solution injected............ 600 cc.
" glucose " .... 30 gm.
" urine excreted............... 499 cc.
" glucose " .... 0 gm.
Per cent of dose.................. 0 per cent.

B. 1.5 gm. per kg. per hr. in 7.5 per cent solution.
Total solution injected........... 600 cc.
" glucose " .... 45 gm.
" urine excreted............. 575 cc.
" glucose " .... 1.02 gm.
Per cent of dose .... 2.27 per cent.

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* This simultaneous break in the sugar and hemoglobin curves is unexplained.

TABLE V.

Dog F6-33.

Weight 12 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
May 22	4.40 p.m.	20	0	0.093	Started injections.
PeriodA.	5.40 "	27	1.93	0.088	
	6.40 "	22	1.37	0.085	6.20 p.m. finished injections.
	7.40 "	32	Faint.	0.115	
	8.40 "	15	"	0.114	Total solution injected..... 180 cc. Total glucose injected..... 36 gm. Total urine excreted....... 86 cc. Total glucose excreted..... 0.82 gm. Per cent of dose......... 2.28 per cent.
June 13	4.30 p.m.	41	0	0.111	Started injections.
PeriodB.	5.30 "	14	2.94	0.170	
	6.30 "	12	2.93	0.132	
	7.30 "	14	Faint.	0.128	7.10 finished injections.
	8.30 "	56	0	0.110	
					Total solution injected..... 270 cc. Total glucose injected..... 54 gm. Total urine excreted....... 96 cc. Total glucose excreted..... 0.76 gm. Per cent of dose......... 1.41 per cent.

TABLE VI.

Dog F6-34.

Weight 7.6 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
June 12	4.30 p.m.	25	0	0.149	Started injections.
	5.30 "	14	3.23	0.208	
	6.30 "	17	3.22	0.176	6.10 p.m. finished injections.
	7.30 "	32	Faint.	0.102	
	8.30 "	20	0	0.103	Total solution injected..... 114 cc. Total glucose injected..... 22.8 gm. Total urine excreted....... 83 cc. Total glucose excreted.... 1.0 gm. Per cent of dose........ 4.40 per cent.

TABLE VII.

Dog F6-33.

Weight 9 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
May 23	4.20 p.m.	25	0	0.109	5.40 p.m. started injections.
	6.40 "	52	3.70	0.159	
	7.40 "	58	2.86	0.152	
	8.40 "	124	2.33	0.218	8.20 p.m. finished injections.
	9.40 "	38	Faint.	0.102	
	10.40 "	12	Very faint.	0.114	Total solution injected..... 203 cc. Total glucose injected..... 40.5 gm. Total urine excreted....... 284 cc. Total glucose excreted..... 6.47 gm. Per cent of dose........ 1.60 per cent.

TABLE VIII.

Dog F6-84.

Weight 9 kg. Intravenous glucose injections, 1 gm. per kg. per hr. (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
May 23	4.35 p.m.	18	0	0.086	5.15 p.m. started injections.
	6.15 "	50	2.63	0.175	
	7.15 "	26	2.80	0.179	
	8.15 "	90	1.50	0.104	7.55 p.m. finished injections.
	9.15 "	63	Faint.	0.075	
	10.15 "	25	Very faint.	0.089	Total solution injected..... 203 cc. Total glucose injected..... 40.5 gm. Total urine excreted....... 244 cc. Total glucose excreted..... 3.41 gm. Per cent of dose......... 8.40 per cent.

TABLE IX.

Dog G7-39.

Weight 11 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1918		*cc.*	*per cent*	*per cent*	
July 10	1.50 p.m.	15	0	0.118	Started injections.
	2.50 "	10	2.00	0.167	
	3.50 "	5	2.80	0.137	
	4.50 "	148	0.26	0.232	4.35 p.m. finished injections.
	5.50 "	44	Very faint.	0.085	
	6.50 "	66	0	0.131	Total solution injected..... 248 cc. Total glucose injected.... 49.5 gm. Total urine excreted....... 273 cc. Total glucose excreted..... 0.73 gm. Per cent of dose......... 1.50 per cent.

TABLE X.

Dog F6-31.

Weight 18 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1918		*cc.*	*per cent*	*per cent*	
April 5	2.30 p.m.	58	0	0.135	Started injections.
	3.30 "	62	0.66	0.182	
	4.30 "	106	0.22	0.132	
	5.30 "	80	0.28	0.192	5.10 p.m. finished injections.
	6.30 "	50	0.59	0.152	
	7.30 "	50	0.12	0.106	Total solution injected..... 405 cc. Total glucose injected.... 81 gm. Total urine excreted...... 348 cc. Total glucose excreted.... 1.22 gm. Per cent of dose .... 1.50 per cent.

TABLE XI.

Dog G7-69.

Weight 8 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1918		*cc.*	*per cent*	*per cent*	
July 10	1.55 p.m.	14	0	0.122	Started injections.
	2.55 "	23	0.65	0.122	
	3.55 "	33	0.48	0.164	
	4.55 "	60	0.23	0.156	4.35 p.m. finished injections.
	5.55 "	56	Very faint.	0.147	
	6.55 "	20	0	0.102	Total solution injected.... 180 cc. Total glucose injected.... 36 gm. Total urine excreted...... 192 cc. Total glucose excreted.... 0.45 gm. Per cent of dose........ 1.28 per cent.

TABLE XII.

Dog F6-32.

Weight 14 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1918		*cc.*	*per cent*	*per cent*	
April 5	2.20 p.m.	72	0	0.120	Started injections.
	3.20 "	46	0.48	0.204	
	4.20 "	47	0.28	0.192	
	5.20 "	132	0.40	0.208	
	6.20 "	70	0.28	0.250	6.05 p.m. finished injections.
	7.20 "	38	0.23	0.109	
					Total solution injected..... 420 cc. Total glucose injected..... 84 gm. Total urine excreted....... 333 cc. Total glucose excreted.... 1.17 gm. Per cent of dose........ 1.40 per cent.

TABLE XIII.

Dog F6-05.

Weight 15 kg. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 10 per cent solution (4 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Dec. 4	10.00 a.m.	50	0	0.109	Started injections.
	11.00 "	148	Faint.	0.118	
	12.00 "	256	Slight.	0.123	
	1.00 p.m.	360	0.18	0.143	
	2.00 "	180	0.78	0.170	
	3.00 "	132	0.32	0.151	3.20 p.m. temperature 39.6°C.
	4.00 "	330	0.14	0.104	
	5.00 "	146	0.19	0.104	6.30 p.m. temperature 39.1°C.
	6.00 "	225	0.25	0.105	6.40 p.m. finished injections.
	7.00 "	146	0.15	0.119	
	8.00 "	40	Very faint.	0.109	Total solution injected..... 2,027 cc. Total glucose injected... 202.7 gm. Total urine excreted... 1,963 cc. Total glucose excreted..... 3.68 gm. Per cent of dose....... 1.88 per cent.

TABLE XIV.

Dog C3-92.

Weight 18 kg. Normal. Continuous intravenous glucose injection, 2 gm. per kg. per hr. in 5 per cent solution.

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
Aug. 4	2.25 p.m.	20	0	0.114	90	2.30 p.m. started injection.
	3.30 "	85	3.00		77	Vomited during experiment 300 cc. of clear fluid, sugar-free.
	4.30 "	90	2.50	0.330	78	
	5.30 "	320	0.50	0.300	77	
	6.30 "	200	Faint.			Finished injection.
	7.30 "	135	0	0.111		
						Total solution injected... 2,160 cc. Total glucose injected... 108 gm. Total urine excreted... 850 cc. Total glucose excreted... 6.4 gm. Per cent of dose..... 5.90 per cent.

TABLE XV.

Dog C3-88.

Weight 10 kg. Non-diabetic; ¼ to ⅓ of pancreas present.* Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	CO_2	Remarks.
		Volume.	Glucose.				
1916		*cc.*	*per cent*	*per cent*	*per cent*	*vol. per cent*	
May 8 Period A.	2.00 p.m.		0	0.117	90		2.02 p.m. injections started.
	2.20 "	4	1.14	0.170	83		
	2.40 "	3	1.82	0.160	90		
	3.00 "	4	2.32	0.208	88		
	3.20 "			0.123	84		
	3.40 "	8	1.85	0.105	86		Low renal threshold.
	4.00 "			0.147			Temperature 102.9°F.
	4.20 "	8	1.47	0.143	82		Note secondary rise of plasma sugar.
	4.40 "	9	1.93	0.164	86		4.42 p.m. finished injections.
	5.00 "	19	0.22	0.122			Temperature 102°F.
	5.30 "	22	Faint.	0.116	90		
	6.30 "	24	Doubtful.				Total solution injected. 150 cc.
	7.15 "	17	0	0.102	87		Total glucose injected... 30 gm. Total urine excreted.. 118 cc. Total glucose excreted.. 0.66 gm. Per cent of dose.... 2.20 per cent.
June 2 Period B.	2.05 p.m.		0	0.133	95		8.00 a.m. fed 500 gm. of lung.
	2.20 "	16	0.51	0.164	74		2.05 p.m. started injections.
	2.40 "			0.164	76		
	3.00 "	18	0.94	0.149	74		
	3.20 "	8	1.10	0.145	85		
	3.40 "	5	1.38	0.167	87		
	4.00 "	15	1.06	0.132	85		
	4.20 "	4	1.54	0.167	90		

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*All operations were performed under ether anesthesia.

TABLE XV—*Concluded.*

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	CO_2	Remarks.
		Volume.	Glucose.				
1916		*cc.*	*per cent*	*per cent*	*per cent*	*vol. per cent*	
June 2 Period B.—Continued.	4.40 p.m.	5	0.88	0.164			4.42 p.m. finished injections.
	5.00 "	15	0.23	0.102			
	5.30 "	92	0	0.087			Total solution injected. 150 cc. Total glucose injected... 30 gm. Total urine excreted... 238 cc.† Total glucose excreted.. 0.60 gm. Per cent of dose.... 2.0 per cent.
	6.30 "	21	0				
	7.15 "	43	0	0.118			
May 25 Period C.	3.00 p.m.		0	0.102	90	55.8	9.30 a.m. fed 200 gm. of lard.
	3.20 "	4	Faint.	0.161	88	54.8	3.00 p.m. started injections.
	3.40 "	6	0.76	0.147	85	50.4	Heavy lipemia.
	4.00 "	3	0.54	0.156	70	54.8	Moderate "
	4.20 "	4	1.24	0.161	65	50.4	
	4.40 "			0.189	74	56.2	Finished injections.
	5.00 "	3	1.37	0.185	74	56.2	
	5.20 "			0.128	65	51.4	Heavy lipemia.
	5.40 "	15	0.26	0.143	62	56.2	" "
	6.00 "	2	1.33	0.143	70	56.2	
	6.30 "	15	Faint.	0.083	75	56.2	Total solution injected.. 90 cc. Total glucose injected.... 20 gm. Total urine excreted. 70 cc. Total glucose excreted... 0.34 gm. Per cent of dose..... 1.70 per cent.
	7.30 "	5	Very faint.				
	8.15 "	13	0	0.100	71	55.8	

† The surplus water excreted is presumably derived from the food.

TABLE XVI.

Dog B2-00.

Weight 14 kg. Partially depancreatized non-diabetic. Two subsequent operations, removing first 0.8 gm. and then 0.1 gm. of pancreas, were required to produce diabetes. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 5 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
Jan. 31	1.30 p.m.		0	0.105	112	1.45 p.m. started injections.
	2.00 "	60	0.36			
	2.20 "	90	0.95	0.288	98	
	2.40 "	100	0.50	0.145	91	3.00 p.m. finished injections.
	3.30 "	175	0	0.091	93	
	3.45 "	25	0	0.088	85	Total solution injected...... 560 cc. Total glucose injected...... 28 gm. Total urine excreted........ 480 cc. Total glucose excreted..... 1.57 gm. Per cent of dose. 5.60 per cent.
	4.00 "	30	0	0.098	80	

TABLE XVII.

Dog B2-01.

Weight 14 kg. Partially depancreatized. Mildly diabetic. Continuous intravenous glucose injections, 1.2 gm. per kg. per hr. in 5 per cent solution.

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 17	2.25 p.m.	25	0	0.116	107	2.30 p.m. started injections.
Period	3.00 "	76	2.44	0.228	103	
A.	4.30 "	190	1.47	0.286	95	Temperature 39.2°C.
	5.00 "	160	1.21	0.238	83	
	5.30 "	95	1.06	0.222	90	Finished injections.
	6.30 "	31	0.34	0.084	99	Note low renal threshold.
	7.10 "	19	Slight faint.	0.087	85	
	8.30 "	69		0.116	90	Total solution injected. 1,008 cc. Total glucose injected.. 50.4 gm. Total urine excreted.. 640 cc. Total glucose excreted.. 7.64 gm. Per cent of dose. 14.1 per cent.
May 24	8.25 a.m.	50	0	0.087	108	Fed 200 gm. of lard.
Period	2.25 p.m.	2	0	0.122	110	3.00 p.m. started injections.
B.	4.00 "	110	1.89	0.385	102	
	5.00 "	216	2.13	0.435	92	
	6.00 "	350	2.04	0.358	97	Finished injections.
	7.00 "	63	1.73	0.182	98	
	8.00 "	10	0.45	0.147	98	Total solution injected. 1,008 cc. Total glucose injected.. 50.4 gm. Total urine excreted.. 758 cc. Total glucose excreted.. 14.97 gm. Per cent of dose. 27.7 per cent.
	9.30 "	7	Faint.	0.147	98	

TABLE XVII—*Continued.*

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 29 Period C.	8.45 a.m.	20	0	0.102	95	Fed 300 gm. of white clay.
	9.45 “			0.120	96	
	2.00 p.m.	2	0	0.103	97	2.30 p.m. started injections.
	3.30 “	74	2.04	0.345	74	
	4.30 “	320	2.38	0.500	66	
	5.30 “	278	3.70	0.385	68	Finished injection.
	6.30 “	30	2.22	0.182		Drank 250 cc. of water.
	7.45 “			0.179		“ 200 “ “ “
	9.00 “	16	0.28	0.135	84	
						Total solution injected. 1,008 cc.
						Total glucose injected.. 50.4 gm.
						Total urine excreted.. 710 cc.
						Total glucose excreted.. 9.84 gm.
						Per cent of dose. 18.2 per cent.
June 1 Period D.	2.40 p.m.			0.104	92	8.00 a.m. fed 200 gm. of lard.
	4.00 “	30	3.65	0.357	88	3.00 p.m. started injections. Slight lipemia.
	5.00 “	348	2.38	0.371	75	Negative “
	6.00 “	182	2.64	0.417	77	Finished injections. Negative lipemia. Vomited about 25 gm. of lard. Drank 200 cc. of water.
	7.00 “	40	4.17	0.193	88	
	8.00 “	22	Faint.	0.145		
	9.30 “	65	0	0.119	77	
						Total solution injected. 1,008 cc.
						Total glucose injected.. 50.4 gm.
						Total urine excreted.. 687 cc.
						Total glucose excreted.. 15.86 gm.
						Per cent of dose. 31.5 per cent.

TABLE XVII—*Concluded.*

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
June 5 Period E.	9.35 a.m.		0	0.093	85	Tantalized with meat for 5 min., not allowed to eat it.
	9.40 "			0.093	87	Fed 500 gm. of lung.
	9.50 "			0.109	82	
	10.10 "			0.119	80	
	10.25 "		0	0.156	78	3.20 p.m. started injections.
	4.20 p.m.	123	3.58	0.358	70	
	5.20 "	232	3.85	0.477	73	
	6.20 "	240	2.18	0.264	72	Finished injections.
	7.20 "	14	0.32	0.122	74	
	8.20 "	19	Faint.	0.182	71	Total solution injected. 1,008 cc. Total glucose injected.. 50.4 gm. Total urine excreted.. 647 cc. Total glucose excreted.. 18.61 gm. Per cent of dose. 37.0 per cent.
	9.50 "	19	"	0.121		

TABLE XVIII.

Dog B2-02.

Weight 10.5 kg. Partially depancreatized. Intravenous glucose injections, 1.5 gm. per kg. per hr. in 5 per cent solution in 0.9 per cent saline (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
Feb. 2	1.55 p.m.		0	0.111	114	2.15 p.m. started injections.
	2.30 "	10	2.32	0.209	108	
	2.50 "	25	2.38	0.271	106	
	3.10 "	35	2.13	0.250	97	2.55 p.m. finished injections.
	3.25 "	12	0.64	0.200	104	
	3.40 "	10	Faint.	0.137	103	Total solution injected.... 316 cc. Total glucose injected.... 15.8 gm. Total urine excreted. 102 cc. Total glucose excreted... 1.66 gm. Per cent of dose..... 10.5 per cent.
	3.55 "	10	0	0.125	99	

TABLE XIX.

Dog C3-74.

Weight 18 kg. Partially depancreatized. Mildly diabetic. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 8 Period A.	3.00 p.m.			0.119	88	Started injections.
	3.20 "	8	1.82	0.173	91	3.05 p.m. blood taken 2 minutes after finishing injection; plasma sugar 0.294 per cent.
	3.40 "	6	3.60	0.263	91	
	4.00 "	19	1.22	0.286	90	
	4.20 "	10	0.86	0.303	94	
	4.40 "	23	0.82	0.312	92	Finished injections.
	5.00 "	22	0.22	0.238	94	
	5.40 "	20	0.31			Total solution injected... 180 cc.
	6.20 "			0.137	96	
	6.40 "	20	Faint.			
	7.00 "			0.126	92	Total glucose injected... 36 gm.
	7.40 "	20	Very faint.			
	8.20 "	20	Very faint.	0.125	93	Total urine excreted. 174 cc. Total glucose excreted... 0.99 gm. Per cent of dose..... 3.02 per cent.
May 23 Period B.	2.10 "		0	0.139	88	10.25 a.m. fed 1,800 gm. of lung.
	2.45 "	20	0.83	0.227	87	
	3.05 "	19	0.95	0.250	87	2.25 p.m. started injections.
	3.25 "	15	1.54	0.264	84	
	3.45 "	18	2.08	0.286	85	
	4.05 "	18	2.38	0.286		Finished injections.
	4.20 "	21	1.93	0.313	86	
	5.00 "	19	0			Total solution injected... 180 cc.
	5.40 "			0.185	88	
	6.00 "	49	0			
	6.20 "			0.139	85	Total glucose injected... 36 gm.
	7.10 "	53	0			
	7.40 "	56	0	0.137	82	Total urine excreted. 288 cc. Total glucose excreted... 1.79 gm. Per cent of dose..... 4.98 per cent.

TABLE XIX—*Concluded.*

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 12 Period C.	2.00 p.m.		0	0.097	90	10.00 a.m. fed 200 gm. of lard.
	2.20 "	13	0.65	0.151	95	2.00 p. m. started injections.
	2.40 "	15	0.70	0.232	85	
	3.00 "	15	0.66	0.162	88	
	3.20 "	18	0.49	0.167	85	
	3.40 "	14	0.61	0.137	88	Finished injections.
	4.00 "	12	0.65	0.107	85	
	4.20 "	10	0			Total solution injected... 180 cc.
	4.40 "	11	0			
	5.20 "	10	0	0.099	83	
	6.20 "	60	0	0.111	78	Total glucose injected... 36 gm.
	7.20 "	190	0	0.115	89	
						Total urine excreted. 368 cc.
						Total glucose excreted... 0.54 gm.
						Per cent of dose..... 1.50 per cent.

TABLE XX.

Dog B2-88.

Weight 13 kg. Mild diabetes. Glycosuria absent on protein-fat diets with addition of 100 gm. of bread; present with addition of bread to 200 gm. Intravenous glucose injections, 1 gm. per kg. per hr. in 5 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1915		*cc.*	*per cent*	*per cent*	
Dec. 2	1.20 p.m.		0	0.118	11.40 a.m. drank 500 cc. of water, also at 1.20 p.m.
	1.59 "	120	0	0.200	1.44 p.m. started injections.
	2.20 "	64	0	0.200	
	2.43 "	73	0	0.232	
	3.05 "	100	0	0.192	
	3.38 "	106	0	0.200	
	3.54 "	64	0	0.200	
	4.15 "	90	0	0.200	
	4.37 "	90	0	0.128	
	4.58 "	110	0	0.112	5.08 p.m. finished injections.
	5.24 "	117	0	0.121	
					Total solution injected........... 860 cc.
					Total glucose injected........... 43.3 gm.
					Total urine excreted........... 934 cc.
					Total glucose excreted........... 0 gm.
					Per cent of dose... 0 per cent.

TABLE XXI.

Dog E5-97.

Weight 14 kg. Mild diabetes. Glycosuria absent on diet of beef lung with 100 gm. of bread; present with addition of bread to 200 gm. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Nov. 1	4.05 p.m.		0	0.095	4.20 p.m. started injections.
	5.20 "	No urine.		0.286	
	6.25 "	70	2.44	0.294	6.10 p.m. finished injections.
	7.30 "	No urine.		0.164	
	8.30 "	40	1.47	0.145	Total solution injected..... 140 cc.
					Total glucose injected..... 28 gm.
					Total urine excreted....... 110 cc.
					Total glucose excreted..... 2.30 gm.
					Per cent of dose. 8.2 per cent.

TABLE XXII.

Dog F6-09.

Weight 20 kg. Mild diabetes. Glycosuria absent on diet of 1 kg. of lung and 100 gm. of bread; heavy on increasing bread to 200 gm. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1918		*cc.*	*per cent*	*per cent*	
Feb. 7	2.15 p.m.		0	0.127	2.25 p.m. started injections.
	3.20 "	25	3.64	0.555	
	4.20 "	87	4.98	0.555	
	5.20 "	180	5.89	0.667	5.10 p.m. finished injections.
					Total solution injected....... 300 cc.
					Total glucose injected....... 60 gm.
					Total urine excreted......... 292 cc.
					Total glucose excreted......... 15.84 gm.
					Per cent of dose.. 26.4 per cent.

TABLE XXIII.

Dog E5-95.

Weight 16 kg. Moderate diabetes. Glycosuria absent on diet of 80 gm. of lung and 100 gm. of suet; present with addition of 50 gm. of bread. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (4 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Nov. 1	1.20 p.m.		0	0.109	1.40 p.m. started injections.
	2.35 "	22	3.62	0.357	
	3.40 "	90	4.31	0.416	3.25 p.m. finished injections.
	4.40 "	32	5.21	0.200	
	5.40 "	10	1.87	0.218	Total solution injected......... 160 cc.
					Total glucose injected......... 32 gm.
					Total urine excreted......... 154 cc.
					Total glucose excreted......... 6.55 gm.
					Per cent of dose. 20.4 per cent.

TABLE XXIV.

Dog D4-62.

Weight 19 kg. Moderate diabetes. Glycosuria absent on diet of 1 kg. of lung; heavy with the addition of 50 gm. of bread. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1916		*cc.*	*per cent*	*per cent*	
Jan. 31	1.45 p.m.		0	0.100	1.50 p.m. started injections.
	2.05 "	10	2.94	0.196	
	2.25 "	21	3.23	0.385	
	2.45 "	25	4.35	0.385	
	3.05 "	21	5.56	0.435	
	3.25 "	28	6.25	0.455	
	3.45 "	27	5.41	0.455	3.50 p.m. finished injections.
	4.05 "	33	4.54	0.416	
	5.05 "	21	3.57	0.384	Total solution injected..... 223 cc.
	6.05 "	4	2.08	0.286	
	7.30 "	7	Faint.	0.137	Total glucose injected..... 44.3 gm.
	10.00 "	10	0	0.088	
					Total urine excreted....... 207 cc.
					Total glucose excreted..... 8.77 gm.
					Per cent of dose......... 19.8 per cent.

TABLE XXV.

Dog D4-84.

Weight 14 kg. Moderate diabetes. Slight glycosuria on 1 kg. of beef lung. Intravenous glucose injections, 1 gm. per kg. per hr. in 10 per cent solution (4 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Oct. 31	1.30 p.m.			0.154	Started injections.
	2.30 "			0.400	
	3.30 "			0.525	Finished injections.
	4.30 "			0.370	
	5.40 "			0.200	Total solution injected......... 280 cc. Total glucose injected......... 28 gm. Total urine, in 24 hrs., 390 cc. with 1.6 per cent glucose. Total glucose excreted......... 6.24 gm. Per cent of dose. 22.2 per cent.

TABLE XXVI.

Dog E5-19.

Weight 9.0 kg. Severe diabetes. Glycosuria absent on diet of 100 gm. of lung and 100 gm. of suet; faint on 200 gm. of lung and 100 gm. of suet; heavy on 300 gm. of lung and 100 gm. of suet. Intravenous glucose injections, 1 gm. per kg. per hr. in 5 per cent solution in 0.9 per cent saline. (4 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Oct. 31	10.15 a.m.		0	0.133	Started injections.
	11.15 "	Not catheterized.		0.322	
	12.15 p.m.	57	5.10	0.475	Finished injections.
	1.15 "	15	3.29	0.278	
	2.15 "	13	2.16	0.286	Total solution injected..... 420 cc. Total glucose injected... 18 gm. Total urine excreted... 85 cc. Total glucose excreted... 3.7 gm. Per cent of dose....... 20.5 per cent. Glucose in 85 cc. of additional urine up to next morning too little to titrate.

TABLE XXVII.

Dog B2-79.

Weight 14 kg. Partially depancreatized. Severe diabetes. Tolerance 500 gm. of lung. Period A, intravenous glucose injections, 1.5 gm. per kg. per hr. in 5 per cent solution in 0.9 per cent saline (3 injections per hr.). Period B, intravenous saline injections, total received 420 cc. (3 injections per hr.).

Date.	Time.	Urine.				Plasma sugar.		Hemoglobin.		Temperature.		Remarks.
		Volume.		Glucose.								
		A	B	A	B	A	B	A	B	A	B	
1915		*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	°*F.*	°*F.*	
Dec. 15	1.30 p.m.	31	50	0	0	0.135	0.167	104	113	101.4	101.6	1.50 p.m. started injections.
Period A.	2.10 "	15	9	1.88	0	0.294	0.147		104	101.1	101.3	
Dec. 27	2.30 "	65	15	2.14	0	0.400	0.139	102	98	100.9	101.7	Finished injections.
Period B.	2.50 "	108	38	2.11	0	0.400	0.139	93	81	100.8	101.6	
	3.10 "	27	13	2.31	0	0.358	0.139	94	81	100.8	101.2	
	3.30 "	13	10	1.78	0	0.250	0.162	85	90	101.1	101.3	
	3.45 "	17	14	0.46	0	0.264	0.167	93	80	101.4	101.4	
	4.00 "	26	20	Very faint.	0	0.208	0.152	90	82	101.4	101.2	
	4.15 "	40	21	0	0	0.226	0.183	96		102.2	101.7	

A. Total solution injected........... 420 cc.
" glucose " .... 21 gm.
" urine excreted............. 311 cc.
" glucose " .... 4.9 gm.
Per cent of dose................ 23.3 per cent.

B. Total solution injected................ 420 cc.
" urine excreted.................. 140 "

TABLE XXVIII.

Dog C3-86.

Weight 13 kg. Partially depancreatized. Severely diabetic. Intravenous glucose injections, 1 gm. per kg. per hr. in 20 per cent solution (3 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 9 Period A.	2.55 p.m.		0	0.128	99	Started injections.
	3.15 "	8	0.57	0.130	95	
	3.35 "	3	1.54	0.200	97	
	3.55 "	10	1.08	0.270	90	
	4.15 "	3	3.45	0.227	84	
	4.35 "	3	2.04	0.232	76	Finished injections.
	4.55 "	4	1.76	0.185	74	
	5.30 "	55	0			Total solution injected..... 130 cc.
	5.45 "			0.123	90	
	7.00 "	91	0	0.099	99	
						Total glucose injected... 26 gm.
						Total urine excreted... 177 cc.
						Total glucose excreted... 0.44 gm.
						Per cent of dose....... 1.69 per cent.
May 15 Period B.						10.30 a.m. fed 200 gm. of lard.
	3.00 "		0	0.098	90	Started injections.
	3.20 "	6	1.79	0.170	90	
	3.40 "	11	0.95	0.170	80	
	4.00 "	11	1.20	0.164	94	
	4.20 "	4	2.15	0.145	100	
	4.40 "	3	2.09	0.137	100	Finished injections.
	5.00 "	5	1.61	0.125	100	
	5.50 "	8	Slight.	0.116	96	Total solution injected..... 130 cc.
	7.00 "	15	0	0.100	89	
	7.30 "	12	0			
						Total glucose injected... 26 gm.
						Total urine excreted... 75 cc.
						Total glucose excreted... 0.57 gm.
						Per cent of dose....... 2.19 per cent.

TABLE XXVIII—*Concluded.*

Date.	Time.	Urine.		Plasma sugar.	Hemoglobin.	Remarks.
		Volume.	Glucose.			
1916		*cc.*	*per cent*	*per cent*	*per cent*	
May 22	11.25 a.m.			0.119	88	Fed 1,200 gm. of lung.
Period	1.50 p.m.	72	0	0.123	88	Started injections.
C.	2.15 "	11	2.38	0.164	88	
	2.35 "	16	2.27	0.185	77	
	2.55 "	9	4.16	0.233	81	
	3.15 "	15	3.85	0.204	80	
	3.35 "	12	3.03	0.200	70	Finished injections.
	3.55 "	12	1.52	0.161	89	
	4.40 "	15	Faint.	0.125	93	4.40 p.m. vomited 400 gm. of undigested lung.
	6.25 "	75	0	0.095	100	
						Total solution injected... 130 cc.
						Total glucose injected.. 26 gm.
						Total urine excreted. 165 cc.
						Total glucose excreted 2.11 gm.
						Per cent of dose....... 8.10 per cent.

TABLE XXIX.

Dog E5-90.

Weight 10 kg. Period A, very mild diabetes. Glycosuria absent on diet of bread and soup; continuously heavy with addition of 100 gm. of glucose. Period B, moderate diabetes. Glycosuria absent on diet of lung and suet; heavy with addition of 200 gm. of bread. Intravenous glucose injections, 1 gm. per kg. per hr. in 10 per cent solution (4 injections per hr.).

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Oct. 25	1.45 p.m.		0	0.164	1.55 p.m. started injections.
Period	2.50 "	5	0.68	0.333	
A.	3.50 "	14	0.57	0.323	4.40 p.m. finished injections.
	4.50 "	95	Faint.	0.213	
	6.15 "			0.119	
					Total solution injected.... 300 cc.
					Total glucose injected.... 30 gm.
					Total urine excreted.... 114 cc.
					Total glucose excreted.... 0.11 gm.
					Per cent of dose........ 0.37 per cent.
Dec. 18	2.45 "		0	0.122	3.00 p.m. started injections.
Period	4.00 "	14	2.25	0.384	
B.	5.00 "	50	4.00	0.500	5.45 p.m. finished injections.
	6.00 "	26	2.60	0.455	
					Total solution injected.... 300 cc.
					Total glucose injected.... 30 gm.
					Total urine excreted.... 90 cc.
					Total glucose excreted.... 3.0 gm.
					Per cent of dose........ 10 per cent.

TABLE XXX.

Dog D4-52.

Weight, Period A, 10.75 kg.; Period B, 11.7 kg.; Period C, 12 kg. Glycosuria absent on bread and soup diet to Oct. 10, 1917. Absent thereafter on 500 gm. of lung and 100 gm. of suet, with hyperglycemia. Intravenous glucose injections, 1 gm. per kg. per hr. in 10 per cent solution (4 injections per hr.), based on normal weight of 12 kg.

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1917		*cc.*	*per cent*	*per cent*	
Aug. 6 Period A.	Before injection.		0	0.218	
	End 1st hr.	16	2.64	0.500	
	" 2nd "	16	5.08	0.715	
	" 3rd "	54	2.84	0.475	Plasma sugar immediately after injection. 0.770 per cent.
	" 4th "	52	1.54	0.590	
	" 5th "	76	0.74	0.415	
	" 6th "	78	1.81	0.270	Total solution injected...... 828 cc.
	" 7th "	94	1.45	0.202	
	" 8th "	82	0.82	0.125	Total glucose injected...... 96 gm.
	1 hr. after injection.	53	0.19	0.128	Total urine excreted........ 563 cc.
	2 hrs. after injection.	12	Faint.	0.130	Total glucose excreted...... 7.8 gm.
	3 hrs. after injection.	29	0	0.133	Per cent of dose. 8.1 per cent.
Nov. 19 Period B.	Before injection.		0	0.169	
	End 1st hr.	36	1.93	0.555	
	" 2nd "	68	3.08	0.555	
	" 3rd "	82	1.49	0.435	
	" 4th "	132	0.79	0.370	
	" 5th "	98	0.39	0.370	Total solution injected..... 1,200 cc.
	" 6th "	126	0.28	0.356	
	" 7th "	127	0.55	0.370	Total glucose injected..... 120 gm.
	" 8th "	105	0.39	0.370	
	" 9th "	105	0.36	0.384	Total urine excreted....... 1,035 cc.
	" 10th "	101	0.42	0.322	
	1 hr. after injection.	45	Faint.	0.156	Total glucose excreted.... 7.7 gm.
	2 hrs. after injection.	10	0	0.147	Per cent of dose........ 6.4 per cent.

TABLE XXX—*Concluded.*

Date.	Time.	Urine.		Plasma sugar.	Remarks.
		Volume.	Glucose.		
1918		*cc.*	*per cent*	*per cent*	
Feb. 19 Period C.	Before injection.		0	0.145	
	End 1st hr.	20	4.77	0.417	
	" 2nd "	55	5.13	0.476	
	" 3rd "	114	3.39	0.500	
	" 4th "	109	3.45	0.525	Total solution injected...... 960 cc.
	" 5th "	102	3.40	0.500	
	" 6th "	140	2.78	0.475	Total glucose injected...... 96 gm.
	" 7th "	90	4.35	0.455	
	" 8th "	87	3.23	0.384	Total urine excreted........ 778 cc.
	1 hr. after injection.	43	0.74	0.294	Total glucose excreted...... 25.7 gm.
	2 hrs. after injection.	19	Very faint.	0.170	Per cent of dose.......... 26.8 per cent.

TABLE XXXI.

Dog D4-69.

Glycosuria absent on bread and soup to Oct. 5, 1917; thereafter absent on 500 gm. of lung and 100 gm. of suet, with development of hyperglycemia. Intravenous glucose injections, 1.3 gm. per kg. per hr. in 10 per cent solution (4 injections per hr.), based on 15 kg. weight.

	Period A. Aug. 6, 1917. Weight 16.2 kg.			Period B. Dec. 4, 1917. Weight 14.2 kg.			Period C. Feb. 19, 1918. Weight 15.5 kg.		
Time.	Urine. Volume.	Urine. Glucose.	Plasma sugar.	Urine. Volume.	Urine. Glucose.	Plasma sugar.	Urine. Volume.	Urine. Glucose.	Plasma sugar.
	cc.	*per cent*	*per cent*	*cc.*	*per cent*	*per cent*	*cc.*	*per cent*	*per cent*
Before injection		0	0.106		0	0.104		0	0.154
End 1st hr	20	1.56	0.278	100	2.84	0.715	46	4.83	0.625
" 2nd "	30	2.87	0.294	144	4.45	0.990	214	4.00	0.800
" 3rd "	64	1.71	0.312	222	3.65	0.990	252	4.35	1.000
" 4th "	106	0.76	0.270	300	2.80	1.000	274	3.70	1.180
" 5th "	110	0.42	0.218	220	2.93	0.844	281	4.25	1.110
" 6th "	76	0.53	0.204	265	2.43	0.844	270	4.55	1.050
" 7th "	128	0.51	0.183	222	2.71	0.625	222	4.55	1.000
" 8th "				216	3.82	0.715	278	5.13	0.950
1 hr. after injection	90	Faint.	0.082	60	3.60	0.370	78	5.88	0.525
2 hrs. " "	82	0	0.098	66	0.49	0.217	10	5.13	0.322

A. Total solution injected 1,560 cc.	B. Total solution injected 1,560 cc.	C. Total solution injected 1,560 cc.
" glucose " 156 gm.	" glucose " 156 gm.	" glucose " 156 gm.
" urine excreted 706 cc.	" urine excreted 805 cc.	" urine excreted 1,925 cc.
" glucose " 4.5 gm.	" glucose " 55.2 gm.	" glucose " 80.4 gm.
Per cent of dose 2.87 per cent.	Per cent of dose 35.4 per cent.	Per cent of dose 51.5 per cent.

TABLE XXXII.

Percentage of Total Intravenously Injected Dose of Glucose Excreted by Normal Dogs.

Dosage per kg. per hr.	1 gm. in 10 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).			1 gm. in 5 per cent solution (2 injections per hr.).	1.5 gm. in 20 per cent solution (3 injections per hr.).								1.5 gm. in 10 per cent solution (4 injections per hr.).	1.5 gm. in 7.5 per cent solution (2 injections per hr.).	2 gm. in 5 per cent solution (continuously).
Duration..................	For 2 hrs.	For 3 hrs.	For 10 hrs.	For 3 hrs.	For 2 hrs.	For 2 hrs.		For 3 hrs.					For 4 hrs.	For 9 hrs.	For 2 hrs.	For 4 hrs.
Table No..................	I	II	III	VIII	IV (A)	V (A)	VI	V (B)	VII	IX	X	XI	XII	XIII	IV (B)	XIV
Per cent of total dose excreted....	0.73	2.16	1.48	8.40	0	2.28	4.40	1.41	1.60	1.50	1.50	1.28	1.40	1.88	2.27	5.90
Per cent of total dose excreted for first 2 hrs.......			0.85											Trace.		
Per cent of total dose excreted for first 3 hrs.......			1.40											0.96		

TABLE XXXIII.

Percentage of Total Dose of Intravenously Injected Glucose Excreted by Partially Depancreatized Dogs.

	No diabetes.		Very mild diabetes.			Mild diabetes.			Moderate diabetes.			Severe diabetes.		
Table No..	XV	XVI	XVII (A)	XVIII	XIX (A)	XX	XXI	XXII	XXIII	XXIV	XXV	XXVI	XXVII	XXVIII (A)
Dosage per kg. per hr.	1 gm. in 20 per cent solution (3 injections per hr.).	1.5 gm. in 5 per cent solution (3 injections per hr.).	1.2 gm. in 5 per cent solution (continuous).	1.5 gm. in 5 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).	1 gm. in 5 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (4 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).	1 gm. in 10 per cent solution (4 injections per hr.).	1 gm. in 5 per cent solution (4 injections per hr.).	1.5 gm. in 5 per cent solution (3 injections per hr.).	1 gm. in 20 per cent solution (3 injections per hr.).
Duration.	For 3 hrs.	For 2 hrs.	For 3 hrs.	For 1 hr.	For 2 hrs.	For $3\frac{1}{2}$ hrs.	For 2 hrs.	For 3 hrs.	For 2 hrs.	For $2\frac{1}{3}$ hrs.	For 2 hrs.	For $2\frac{1}{4}$ hrs.	For 1 hr.	For 2 hrs.
Per cent of total dose excreted.	2.2	5.6	14.1	10.5	3.02	0	8.2	26.4	20.4	19.8	22.2	20.5	23.3	1.69

ALKALI RESERVE OF SWINE AS AFFECTED BY CEREAL FEEDING AND MINERAL SUPPLEMENTS.

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It is common knowledge among students of animal nutrition that cereal rations are characterized by excess of acid as compared with basic mineral elements, and by a marked deficiency of calcium. A question remains, however, as to whether this potential acidity of cereal rations is of such degree as to constitute it a practical consideration, and especially as to the extent to which this acidity should be considered as merely incidental to the calcium shortage.

In the investigation of the physiological significance of the potential acidity of cereal rations it becomes a matter of much interest to determine the influence of this factor in relation to the composition and functional efficiency of the blood and tissues. A significant and fundamental matter in this connection is the maintenance of the alkali reserve, which may be considered, in general terms, as composed of those constituents of the body which can be used for acid neutralization. In the method of estimation of Van Slyke, Stillman, and Cullen,[1] which was used in this study, the alkali reserve is determined as the bicarbonate concentration of the blood plasma.

Ammonia and acidity were estimated by the methods of Folin, and hydrogen ion concentration by the colorimetric method of Clark and Lubs, using the colored screen and the comparator.

The blood samples were taken from the tail, the handling of the subjects being successfully accomplished by the use of a crate devised for hog-cholera serum work. The tail was shaved, the end clipped off, and the blood drawn through a 5 cm. funnel into a 50 cc. centrifuge tube where it was collected under a 0.5 cm. layer of liquid petrolatum (Squibb). The funnel was fitted to the tube with a two-hole rubber stopper, and the funnel and tube together contained powdered potassium oxalate equal to 0.1 per cent of the blood drawn. The tail was held inside the funnel, with the bleeding tip within 1 or 2 cm. of the bottom, to minimize possible loss of carbon dioxide.

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[1] Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1919, xxxviii, 167.

In this study two pigs were fed a cereal ration composed of corn 7 parts, wheat middlings 1 part, and linseed oil meal 1 part, with salt (NaCl) weighed separately at a rate of 2.2 gm. per kilo of feed. The two pigs weighed 65.0 and 62.4 kilos, respectively, on October 14, at the beginning of the experimental treatment, and on January 2, 112.3 and 110.0 kilos, respectively. The experiment terminated on January 3. The pigs were fed in metabolism crates, and cared for as in metabolism experiments.

In the first period the cereal ration was fed alone for 33 days, during the last 7 of which alkali reserve estimations on the blood plasma were made twice on each subject.

In the second period there was then added to the cereal ration calcium carbonate in amounts equivalent to 200 mg. of calcium per kilo of live weight of the pigs. This treatment was continued for 25 days, during the last 12 of which alkali reserve estimations were made on the two subjects three and four times, respectively, at intervals as shown in Table I.

In the third period the mineral supplement was changed to precipitated bone phosphate, which is largely in the dicalcic form, and which was fed in amounts furnishing, as before, 200 mg. of calcium per kilo of live weight. This treatment was continued for 23 days, during the last 13 of which alkali reserve estimations were made four times on each subject. The condensed data of the experiment will be found in Table I. Alkali reserve estimations were made on the days indicated, while estimations of urinary acid, ammonia, and hydrogen ion concentration were made daily during the greater part of the study and on alternate days during the remainder.

The data show that by the addition of calcium carbonate to a cereal ration the alkali reserve of the blood plasma was increased 10.1 and 10.8 per cent, with the two pigs, above that which pre-

TABLE I.

Effects of Cereal Ration and Mineral Supplements on Blood Plasma and Urine of Swine.

Pig No.	Treatment.	Date of estimation.	Blood plasma.		24 hr. urine.		
			Molecular concentration of CO_2 as bicarbonate.	CO_2 as bicarbonate.	Acidity.	Ammonia.	H ion concentration.
		1919		*vol. per cent*	*cc. 0.1 N*	*cc. 0.1 N*	*pH*
1	Basal ration of cereals...............	Nov. 11	0.0297	66.5			
1	Basal ration of cereals...............	" 17	0.0314	70.3			
Average..........................			0.0306	68.4	255 (8)[*]	4.18 (8)	7.1 (8)
1	Calcium carbonate...	Nov. 29	0.0338	75.7			
1	" " ...	Dec. 3	0.0354	79.3			
1	" " ...	" 9	0.0327	73.2			
1	" " ...	" 11	0.0328	73.5			
Average..........................			0.0337	75.4	−49 (13)	1.71 (13)	7.7 (13)
1	Precipitated bone phosphate..........	Dec. 22	0.0269	60.3			
1	Precipitated bone phosphate..........	" 26	0.0282	63.2			
		1920					
1	Precipitated bone phosphate..........	Jan. 2	0.0295	66.1			
1	Precipitated bone phosphate..........	" 3	0.0303	67.9			
Average..........................			0.0287	64.4	705 (15)	4.01 (15)	6.36 (15)
		1919					
2	Basal ration of cereals...............	Nov. 12	0.0323	72.3			
2	Basal ration of cereals...............	" 17	0.0323	72.4			
Average..........................			0.0323	72.4	638 (8)	4.10 (8)	6.5 (8)

[*] The numbers in parentheses indicate the number of daily estimations averaged.

TABLE I—*Concluded.*

Pig No.	Treatment.	Date of estimation.	Blood plasma.		24 hr. urine.		
			Molecular concentration of CO_2 as bicarbonate.	CO_2 as bicarbonate.	Acidity.	Ammonia.	H ion concentration.
		1919		*vol. per cent*	*cc. 0.1* N	*cc. 0.1* N	*pH*
2	Calcium carbonate...	Nov. 29	0.0350	78.4			
2	" " ...	Dec. 3	0.0355	79.5			
2	" " ...	" 9	0.0370	82.9			
Average.........................			0.0358	80.3	−29 (13)	1.06 (13)	7.6 (13)
2	Precipitated bone phosphate.........	Dec. 22	0.0306	68.5			
2	Precipitated bone phosphate.........	" 26	0.0314	70.3			
		1920					
2	Precipitated bone phosphate.........	Jan. 2	0.0281	62.9			
2	Precipitated bone phosphate.........	" 3	0.0313	70.1			
Average.........................			0.0303	68.0	1,707 (15)	3.89 (15)	5.57 (15)

vailed during the feeding of the cereal ration alone; and that then, by the substitution of precipitated bone phosphate for the calcium carbonate, the alkali reserve was reduced 14.8 and 15.4 per cent, with the two subjects, to figures which are distinctly lower, in each case, than those obtained from the same individual on the cereal ration. The estimations of acidity, ammonia, and hydrogen ion concentration in the urine vary, as affected by the mineral supplements, in a manner concordant with the alkali reserve estimations, which they serve to confirm. The separate estimations of urinary acidity reveal marked daily and enormous individual variation in the details of the eliminative function.

The alkali reserve of the blood plasma of swine, therefore, is susceptible of variation, and may be either increased or decreased by the use of mineral supplements added to a cereal ration in such quantities as might be used in practical feeding. Since these variations would in all probability be followed, in time, by variations in the other alkali reserves of the body, it is more than likely that changes of body function would result. These might be favorable or unfavorable in accord with the ration and mineral supplements used.

In this connection we are unable to follow Lamb and Evvard[2] in some of the conclusions which they draw from their study of the acid-base balance in swine. Thus, contrary to their main conclusion, "On neither ration did the mineral acid cause a significant loss of calcium, nor did it interfere with the storage of protein," their data for Experiment 1 show, as a result of mineral acid ingestion, a decreased retention of calcium amounting to one-fourth or one-fifth according to whether it is computed with reference to the *absolute amount* or the *proportion of the intake* retained. It should also be noted that the results from Experiment 2 are conflicting since the first control period makes it appear that the mineral acid feeding had *diminished* the loss of calcium by 0.030 gm. of CaO per day, while the second or following control period indicated, with opposite significance, that the acid feeding had *increased* the loss by 0.550 gm. of CaO per day. The authors averaged these contrary findings.

Further, their finding that the mineral acid ingestion had not affected the hydrogen ion concentration of the blood was beside the point, since this constant would increase only as death was about to supervene; and their evidence as to the effect of acid ingestion on the reproductive activity of the sows lacks conclusiveness since only one pig was raised out of eleven farrowed.

We believe therefore that the ultimate effects of the potential acidity of cereal rations on the functional activities of swine remain yet to be determined.

CONCLUSION.

The alkali reserve of the blood plasma of swine may be significantly increased by the feeding of the potentially basic precipitated calcium carbonate, or decreased by the feeding of the potentially acid precipitated calcium phosphate, when these substances are fed as supplements to a cereal ration in quantities such as might be used in practical feeding.

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[2] Lamb, A. R., and Evvard, J. M., *J. Biol. Chem.*, 1919, xxxvii, 317.

THE OCCURRENCE OF WATER-SOLUBLE VITAMINE IN SOME COMMON FRUITS.*

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Despite the fact that the edible fruits enter liberally into the dietary of man in nearly every part of the world, they have hitherto received almost no serious consideration at the hands of scientific investigators. Usually they are casually grouped for consideration with the familiar vegetables with which, it may be noted, they have many features of composition in common. Like these vegetables most fruits contain considerable water; some of them furnish a liberal amount of sugar or starch; and they cannot be classed as significant sources of fat or protein. One may read in government leaflets that

> "fruits and vegetables are necessary for health because they supply certain needed substances that are not usually found in any other food materials. They should have a place in the diet of all those who have passed babyhood and no pains should be spared to obtain at least a small amount of them every day."[1]

The reason for such advice is found in the statement that

> "fruits and vegetables are used in the diet to give pleasant flavor and varied texture. They are important not only for this but because they give bulk and are laxative; because they contain valuable mineral salts, such as

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* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

[1] The day's food in war and peace, U. S. Food Administration, U. S. Dept. Agric. leaflet, 75.

lime and iron; and because they furnish the dietary essentials sometimes called vitamines."[2]

The explanation of the asserted laxative action of the fruits is sought in part in their content of "certain mild vegetable acids useful in preventing constipation." A recent review summarizes the status of fruits in these words:

"No thorough studies of the dietary properties of fruits have yet been made, but from their known chemical composition and biological functions as storage organs, their proper place in the diet can be predicted. They are good sources of mineral salts and of energy-yielding foods, the sugars. They are highly palatable and exert a favorable influence on the excretory processes of the kidneys and the intestine. Their liberal use in the diet should be encouraged."[3]

In so far as the content of inorganic constituents is concerned it must be noted that the edible portions of the fruits and the quantities in which they are customarily eaten in general furnish comparatively little of those elements, notably calcium and phosphorus, which alone deserve more serious consideration in the selection of the dietary.[4] Hence they merit less attention from the standpoint of their "mineral nutrients." It has long been appreciated, however, that some of the fruits have a potency, formerly quite inexplicable, in preventing the onset of scurvy under the conditions now known to permit its appearance. Since the demonstration that this malady is to be classed with the deficiency diseases, the rôle of fruits as antiscorbutics has been widely investigated. The potency of the juice and inner peel of the orange, the juice of the lemon, and some of the rarer fruits, such as the tamarind, cocum, and mango,[5] has been established experimentally on animals and man. On the other hand the antiscorbutic value of the West Indian lime, *Citrus medica acida*, long cherished by the public, has been demonstrated to be insignificant in comparison with the efficacy of other citrus fruits, *Citrus medica limonum*.[6]

[2] The day's food in war and peace,[1] p. 76.

[3] McCollum, E. V., The newer knowledge of nutrition, New York, 1919, 142.

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 131.

[5] Chick, H., Hume, E. M., and Skelton, R. F., *Lancet*, 1919, ii, 320.

[6] Chick, H., Hume, E. M., and Skelton, R. F., *Lancet*, 1918, ii, 735.

According to Lewis[7] the banana contains some antiscorbutic vitamine. Hess and Unger[8] concluded from their investigations on scorbutic infants that "the banana possesses but mild antiscorbutic virtue" when compared to other commonly used fruits.

With respect to the distribution of the other vitamines—the so called fat-soluble A and water-soluble B—now recognized as physiologically distinct accessory food factors, almost no experimental data are available. In the case of the banana Loeb and Northrop[9] have found that under aseptic conditions the fruit fly, *Drosophila ampelophila*, cannot be raised upon bananas, but will grow when yeast is supplied in addition. This suggests the possibility of a deficiency of the banana in the water-soluble vitamine —a conclusion also reached by Sugiura and Benedict[10] from experiments on rats.

Harden and Zilva[11] have concluded, as a result of a study of the action of adsorbents upon autolyzed yeast and orange juice (representing sources of antineuritic and antiscorbutic vitamine, respectively) and mixtures of them, that the two vitamines are not identical, because the potencies characteristic of them could be separated by fullers' earth or dialyzed iron. Incidentally these investigators state: "we have so far not come across a natural product which contains both [antineuritic and antiscorbutic factors] in quantities suitable for investigation" (p. 100). The implication thus remains that orange juice exhibits only the antiscorbutic quality in noteworthy amounts.[12]

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[7] Lewis, H. B., *J. Biol. Chem.*, 1919, xl, 91.

[8] Hess, A. F., and Unger, L. J., *Am. J. Dis. Child.*, 1919, xvii, 221.

[9] Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1916, xxvii, 309. Northrop, J. H., *J. Biol. Chem.*, 1917, xxx, 181.

[10] Sugiura, K., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxvi, 171.

[11] Harden, A., and Zilva, S. S., *Biochem. J.*, 1918, xii, 93.

[12] In a paper which has appeared since this manuscript was prepared, Byfield, Daniels, and Loughlin have reported that in experiments on babies and on rats "growth, as evidenced by the weight curves, was in all cases stimulated when orange juice was given. On the other hand, orange juice from which the antineuritic vitamin has been removed was without influence." To these investigators "it appears that the appetite factor plays only a minor rôle in the stimulating effect of the water soluble vitamin on growth, provided a nearly adequate amount is being given." They also conclude that "Orange juice from which the antineuritic vitamin is

Experiments with Fruits as Sources of Vitamines.[13]

Methods of Feeding.—The routine customary in our laboratory in nutrition experiments on albino rats has been followed in this investigation of the fruits. The standard food mixture consisted of

	per cent
Meat residue	19.6
Salt mixture*	4.0
Starch	52.4
Butter fat	9.0
Lard	15.0

*For composition of salt mixture see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

This was selected, in accord with earlier experience,[14] to furnish a diet free from water-soluble vitamine (B). The possible presence of the latter in the various fruits was tested for by furnishing daily a measured amount of the fresh fruit, the fruit juice, or dried products thereof apart from the rest of the ration. In a few cases the addendum was incorporated in the food mixture, but as a rule it was supplied separately so as not to alter the flavor or palatability of the standard diet. The effects of the presence or removal of the fruit products on nutrition as expressed in food intake and body weight are shown in selected tables and charts in the appendix.

Orange.—The fresh juice, prepared by squeezing the skinned oranges, *i.e.* the edible portion,[15] contains sufficient water-soluble vitamine to promote growth in rats at about the same rate as do

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removed by adsorption does not stimulate growth. This would seem to indicate that the antiscorbutic vitamin lacks growth-stimulating properties" (Byfield, A. H., Daniels, A. L., and Loughlin, R., *Am. J. Dis. Child.*, 1920, xix, 349).

[13] A preliminary notice of some of the results obtained was published by Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1919, xvii, 46.

[14] Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.

[15] Data regarding this are given by Joslin, E. P., The treatment of diabetes mellitus, Philadelphia and New York, 2nd edition, 1917, 265.

the same volumes of milk.[16] This is apparent from Charts I and II in which the effects of varying daily doses (2.5 cc., 5 cc., 10 cc.) of orange juice as the source of water-soluble vitamine upon the growth of rats are shown. In our similar experiments with cow's milk a daily intake of at least 10 to 15 cc. was needed to produce growth at a rate approaching the average. Illustrative data regarding food intakes in the orange experiments are summarized in Table I. The fall in body weight accompanying the decline in food intake when the feeding of orange juice was discontinued is indicated in Chart I (Rats 6106, 6127, 6141) and in Chart II (Rats 6333, 6338), as well as the characteristic restoration following renewed administration of this fruit product. That the advantage of the latter is attributable merely to increased calories (sugar, fruit acids, etc.), *i.e.* to supplementing the basal diet, is not a tenable assumption.

Orange juice was desiccated by preliminary concentration *in vacuo* and drying upon starch in a current of warm air. This dry product,[17] fed in quantities equivalent to measured portions of fresh orange juice, was apparently of equal potency as a source of water-soluble vitamine (B) (Chart II). Givens and McClugage[18] have shown that the antiscorbutic properties of orange juice are likewise not lost by suitable desiccation.

Inasmuch as it has been found by Hess and his associates[19] that the inner peel of the orange has considerable antiscorbutic potency we tested this product both fresh and dried for water-soluble B. When the rats would eat the peel, doses representing 5 gm. per day were quite efficacious (Chart III). The failure to grow vigorously, which is shown during the earlier periods in the records of Rats 6134, 6137, and 6138, is associated with the refusal to consume entirely the portions of peel offered to them daily.

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[16] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 537; *J. Biol. Chem.*, 1920, xli, 515. In their recently published observations upon the growth-promoting effects of orange juice on rats, Byfield, Daniels, and Loughlin[12] showed that, with a ration essentially like our standard diet, 55 cc. of orange juice per 100 gm. of food were insufficient to promote maximal growth, 75 cc. per 100 gm. being more efficacious. Our own data are given in Table I.

[17] We have lately fed such materials in the form of tablets compressed with a machine which secures accurate regulation of dosage.

[18] Givens, M. H., and McClugage, H. B., *Am. J. Dis. Child.*, 1919, xviii, 30.

[19] Hess, A. F., and Fish, M., *Am. J. Dis. Child.*, 1914, viii, 385. Hess, A. F., and Unger, L. J., *J. Biol. Chem.*, 1918, xxxv, 487. Hess, A. F., *J. Am. Med. Assn.*, 1918, lxxi, 941.

Lemon.[15]—The procedure with lemon juice was essentially like that for the orange, dried products being tested. The quantitative relations are about the same, quantities less than 10 cc. of juice per day being insufficient as sources of the water-soluble vitamine for growing rats (Chart IV).

Grapefruit.[15]—Desiccated juice prepared from the edible portion was used in quantities equivalent to 10 cc. of the fresh juice (Chart V). It contains water-soluble vitamine.

Grape.—A commercial grape juice (Welch's) used in 10 cc. doses of the fluid or its equivalent desiccated on starch was tested (Chart VI). Compared volume for volume these products were far less potent than orange, lemon, or grapefruit juices.

Apple and Pear.—In the case of these fruits their bulky character made it practically impossible to feed to a rat more than 10 gm. of the fresh product per day without a reduction of the essential basal ration to a quantity incompatible with maintenance. That some water-soluble vitamine is furnished by apples and pears is shown by the rapid declines when these are withdrawn from the otherwise adequate diet (Charts VII and VIII). In several cases the superior potency of 0.1 to 0.2 gm. of dried brewery yeast per day is shown by contrast. A few preliminary tests with the juice expressed from 10 gm. of apple showed no advantage over the more bulky fresh fruit (Chart VII).

Prune.—Tests with prunes showed that, fed in 2 and 5 gm. daily portions, they are not devoid of water-soluble vitamine (B); the gains made were decidedly greater than those exhibited in the experiments with apples and pears (Chart IX).

Do the Fruits Contain the Fat-Soluble Vitamine?

Our experiments to secure an answer to this question are not yet completed. The indications are, however, that the juices of the lemon and grapefruit respectively, fed in the equivalent of 10 cc. daily doses, do not contain sufficient fat-soluble vitamine to avert the decline in body weight or to heal the eye disease (xerophthalmia, keratomalacia) which may ensue when animals are kept for some time on a diet devoid of the fat-soluble factor. Since as little as 0.1 gm. of butter fat per day may suffice to restore these animals to health it seems unlikely at the present stage of our investigation that the juices of these fruits contain more than traces, if any, of the fat-soluble vitamine. In the case of the orange, preliminary tests indicate that a supply of dried juice has averted or at least delayed the symptoms just referred to as characteristic of a régime lacking fat-soluble vitamine. The observations are as yet too limited in number to permit a final decision on this point.

SUMMARY.

The fresh juices of the edible parts of the *orange*, *lemon*, and *grapefruit* contain water-soluble (B) vitamine. Their potency in this respect is quite similar to that of comparable volumes of cow's milk. The efficiency of these fruit juices is not lost by suitable modes of desiccation. A sample of *grape* juice tested was less potent than equal volumes of the fruit juices just mentioned.

The edible portions of *apples* and *pears* furnish some water-soluble vitamine; the quantity of these fruits necessary to supply this dietary essential is relatively very large, so that from a comparative standpoint they cannot be regarded as rich in this food factor. *Prunes* apparently are richer in the water-soluble vitamine.

From preliminary experiments it seems doubtful whether the juices of the lemon or grapefruit contain more than traces, if any, of the fat-soluble vitamine. Our preliminary observations upon orange juice are indicative of some potency in this vitamine.

The experiments with fruits place the dietary value of these foods, hitherto recommended because of their salt content, their laxative properties, or their antiscorbutic potency, in a new light as sources of water-soluble vitamine.

EXPLANATION OF TABLES.

The data here summarized indicate the successive weekly intakes (in gm.) of the basal ration by the rats for which the changes in body weight are represented graphically in the appended charts. The daily dose of the various fruit products (in equivalent cc. or gm.) is shown in bold-faced type at the beginning of each period. The figures may be studied in connection with the corresponding charts.

TABLE I.

Orange Juice (Charts I and II).

Rat 6141♂.	Rat 6127♂.	Rat 6106♂.	Rat 6256♀.	Rat 6338♂.	Rat 6333♂.	Rat 6427♂.	Rat 6428♂.	Rat 6375♂.	Rat 6373♂.	Rat 6372♂.
gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
5 cc.	**5 cc.**	**5 cc.**	**None.**	**10 cc.**	**10 cc.**	**5 cc.**	**5 cc.**	**5 cc.**	**5 cc.**	**5 cc.**
53	57	64	33	32	35	44	46	31	47	35
(?)	(?)	(?)	28	44	40	44	37	42	47	43
10 cc.	**10 cc.**	**10 cc.**	(?)	52	41	21	19	36	**2.5 cc.**	**2.5 cc.**
42	49	48	**5 cc.**	62	47	42	39	29	41	29
39	50	46	44	53	47	50	30	27	37	30
47	56	50	50	59	46	28	26	30	28	25
51	48	46	45	49	48	33	33	22	20	24
51	50	46	41	**None.**	**None.**	37	37	18	27	24
54	47	51	33	38	38	37	36	23	28	21
(?)	54	56	40	21	37	**10 cc.**	**10 cc.**	28	28	27
65	45	54	51	(?)	26	57	44	26	28	20
60	54	54	39	**5 cc.**	**5 cc.**	65	54	23	22	24
63	59	59		40	31			**10 cc.**	17	24
56	54	57		27	29			34	**15 mg.**	**15 mg.**
48	54	49		31	29			20	**yeast**	**yeast**
None.	**None.**	**None.**		37	25			32	**fraction.**	**fraction.**
30	32	41		28	22				47	39
22	35	26		29	32				46	38
10 cc.	37	(?)		34	25				53	42
56	**10 cc.**	33		36	**10 cc.**					
67	56	29		**10 cc.**	34					
55	60	31		41	39					
	64	26		53	50					
	42	24								
	47	27								
	48	**5 cc.**								
	0.2	42								
	gm.	46								
	yeast.	41								
	75	45								
	65	36								
		39								
		34								
		42								
		None.								
		26								
		14								
		Dead.								

TABLE II.

Orange Inner Peel (Chart III).

Rat 6352♂.	Rat 6354♂.	Rat 6138♂.	Rat 6137♂.	Rat 6134♂.
gm.	*gm.*	*gm.*	*gm.*	*gm.*
5 gm.	**5 gm.**	**5 gm.**	**5 gm.**	**5 gm.**
(?)	(?)	54	60	51
48	37	53	54	45
55	43	45	48	38
42	37	36	37	33
47	34	44	44	37
37	32	37	56	42
32	33	40	55	39
31	36	40	47	40
29	51	45	51	53
16	46	44	51	52
36	48	46	39	46
37	48	64	47	49
34	58	69	**None.**	**None.**
25	52	70	34	22
30	56	62	(?)	19
33	57	41	**0.4 gm. yeast.**	Dead.
None.	**None.**	59	69	
27	31	64	82	
Dead.	24	58		
		58		

TABLE III.

Lemon Juice (Chart IV).

Rat 6325♂.	Rat 6324♂.	Rat 6329♂.	Rat 6441♂.	Rat 6442♂.
gm.	*gm.*	*gm.*	*gm.*	*gm.*
5 cc.	**5 cc.**	**5 cc.**	**2.5 cc.**	**2.5 cc.**
27	25	25	31	28
33	36	32	38	30
35	30	37	27	33
10 cc.	**10 cc.**	**10 cc.**	27	27
44	40	48	27	Dead.
57	35	(?)	27	
56	41	54	**10 cc.**	
46	40	52	29	
59	45	57		
None.	**None.**	**None.**		
42	39	(?)		
22	19	37		
22	15	(?)		
5 cc.	**5 cc.**	**5 cc.**		
44	40	40		
42	29	35		
46	34	30		
41	27	40		
39	35	37		
36	28	34		
40	34	31		
35	30	30		

TABLE IV.

Grapefruit (Chart V).

Rat 6396♂.	Rat 6399♂.	Rat 6394 ♂.
gm.	*gm.*	*gm.*
10 cc.	**10 cc.**	**10 cc.**
35	45	38
47	50	38
55	41	43
58	50	45
49	38	45
50	44	31
48	43	23
42	33	31
36	42	33
35	38	32
33	34	17
51	**None.**	34
60	28	35
56	30	38
	36	

TABLE V.

Grape (Chart VI).

Rat 6381♂.	Rat 6377♂.	Rat 6380♂.	Rat 6336♀.	Rat 6337♀.
gm.	*gm.*	*gm.*	*gm.*	*gm.*
10 cc.	10 cc.	10 cc.	None.	None.
38	41	37	29	29
33	32	41	32	33
43	46	43	20	18
35	33	25	10 cc.	10 cc.
35	39	27	20	24
35	33	30	25	18
31	26	29	23	32
35	40	23	22	28
34	45	30	28	26
28	40	33	29	20
27	29	29	29	21
30	33	23	31	33
None.	35	None.	29	25
(?)	38	24	24	26
(?)		22	37	18
(?)		27	29	26
			21	22
			31	28
			None.	None.
			18	17
			20	22
			Dead.	Dead.

TABLE VI.

Apple (Chart VII).

Rat 5986♂.	Rat 5946♂.	Rat 5974♂.	Rat 5971♂.	Rat 6135♂.	Rat 6129♂.	Rat 6133♂.	Rat 6340♂.	Rat 6339♂.
gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
4 gm.	None.	None.	None.	**5 gm.**	**5 gm.**	**5 gm.**	**5 cc.**	**5 cc.**
57	(?)	38	58	(?)	(?)	(?)	30	22
56	43	(?)	30	40	41	39	39	29
42	26	**4 gm.**	(?)	31	38	28	34	28
10 gm.	**4 gm.**	38	**4 gm.**	**10 gm.**	(?)	**10 gm.**	26	28
31	24	**8 gm.**	20	29	**10 gm.**	20	22	25
43	(?)	34	**8 gm.**	40	36	27	(?)	**10 cc.**
35	**0.2 gm.**	36	35	28	28	24	**10 cc.**	29
32	**yeast.**	**10 gm.**	32	38	37	28	22	20
41	51	34	**10 gm.**	32	25	26	**0.1 gm.**	**0.1 gm.**
39	(?)	45	28	33	28	24	**yeast.**	**yeast.**
34	**10 gm.**	41	32	29	41	19	31	44
36	45	39	34	36	37	Dead.	41	43
38	50	37	29	None.	None.		49	39
33	37	31	31	24	Dead.			
0.2 gm.	34	28	23	Dead.				
yeast.	31	36	27					
79	31	33	27					
54	31	34	30					
53	51	**0.2 gm.**	29					
	37	**yeast.**	**0.2 gm.**					
	36	73	**yeast.**					
	None.	62	71					
	49	66	51					
	22		50					
	(?)							
	0.2 gm.							
	yeast.							
	68							
	52							
	52							

TABLE VII.

Pear (Chart VIII).

Rat 6254♂.	Rat 6240♂.	Rat 6139♂.	Rat 6122♂.	Rat 6131♂.
gm.	*gm.*	*gm.*	*gm.*	*gm.*
10 gm.	**10 gm.**	**5 gm.**	**5 gm.**	**5 gm.**
35	31	(?)	(?)	(?)
45	34	34	49	37
46	36	29	33	27
36	32	29	**10 gm.**	24
41	39		33	20
34	37		27	32
22	37		27	30
0.2 gm. yeast.	**None.**		32	30
60	15		38	31
54	(?)		44	31
64	**0.2 gm. yeast.**		42	32
	39		44	29
	42		38	**10 gm.**
	44		31	25
			0.2 gm. yeast.	31
			83	32
			73	32
			67	**None.**
				25
				Dead.

TABLE VIII.

Prune (Chart IX).

Rat 6477♂.	Rat 6474♂.	Rat 6458♂.	Rat 6478♂.	Rat 6429♂.	Rat 6449♂.
gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
2 gm.	**2 gm.**	**2 gm.**	**5 gm.**	**5 gm.**	**5 gm.**
29	22	26	19	43	31
44	37	39	49	48	46
53	46	47	**1 gm.**	48	42
	38	38	51	27	33
	57	36		48	34
				53	34
				46	55
				48	
				41	
				2 gm.	
				34	
				48	

EXPLANATION OF CHARTS.

In the following charts showing changes in body weight the uninterrupted lines represent the periods during which the indicated quantities of the various fruits, estimated on the fresh basis, were fed daily. During the periods represented by the dotted line (- - - - -) no water-soluble vitamine whatever was fed. In a number of the experiments dried brewery yeast was used for comparison as a source of the water-soluble vitamine during the periods represented by the interrupted line (-·-·-·-). The rapid restoration of weight when fruits have been used as the source of water-soluble vitamine after a period of decline in body weight upon rations devoid of this food factor is characteristic of what we have previously termed the "curve of repair." Under these circumstances quantities of the fruits which are insufficient during the ordinary course of growth to promote the maximum increment of weight may suffice to secure rapid restoration in size.

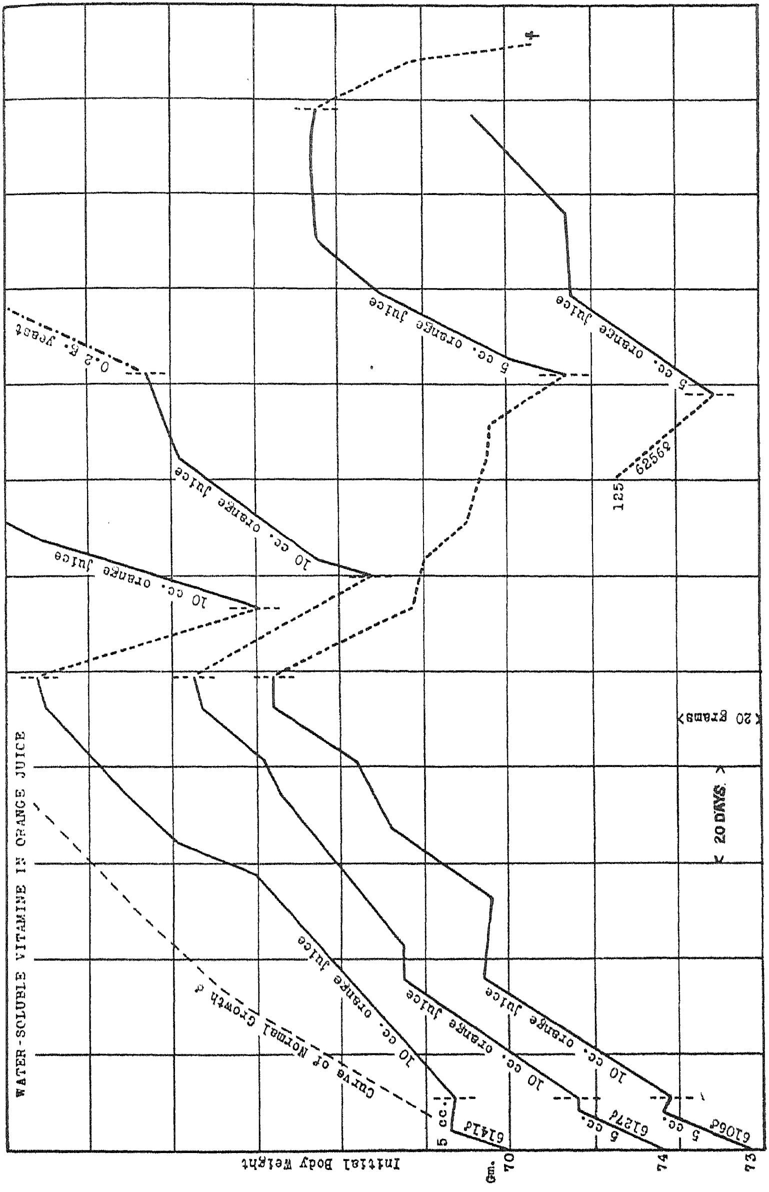
WATER-SOLUBLE VITAMINE IN ORANGE JUICE
Curve of Normal Growth ♂
10 cc. orange juice
10 cc. orange juice
10 cc. orange juice
10 cc. orange juice
10 cc. orange juice
5 cc. orange juice
5 cc. orange juice
0.2 g. yeast
5 cc.
5 cc.
5 cc.
6141♂
6127♂
6106♂
6256♀
125
+
20 DAYS
20 grams
Initial Body Weight
Gm.
70
74
73

CHART I.

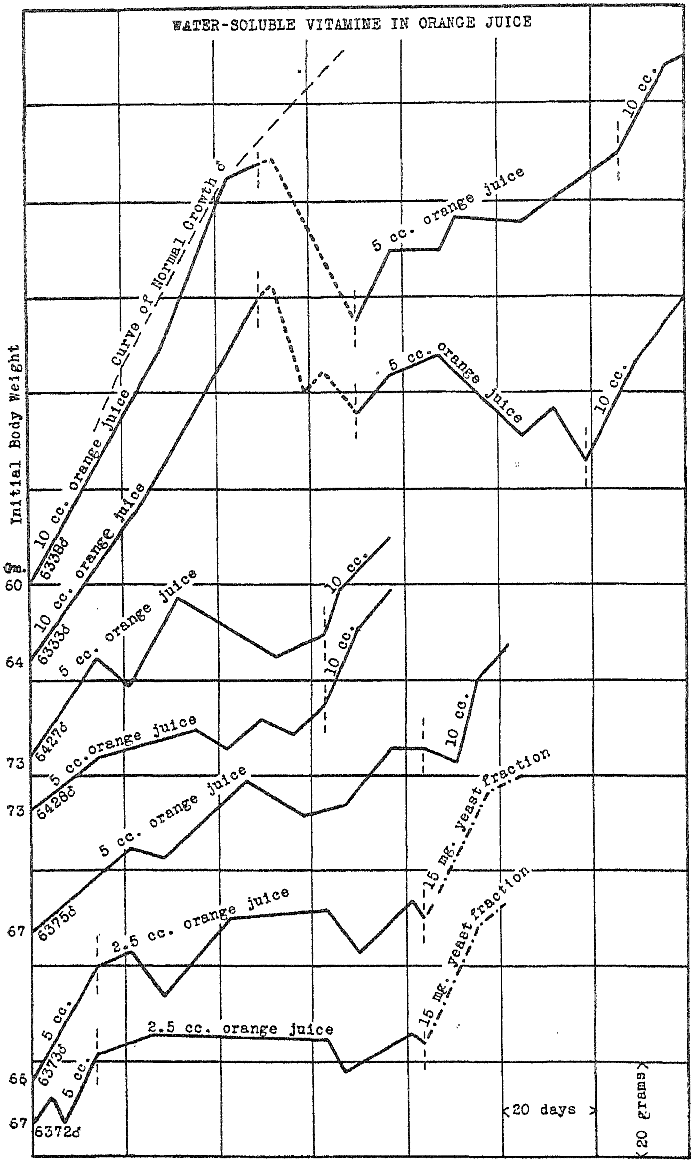
WATER-SOLUBLE VITAMINE IN ORANGE JUICE
Initial Body Weight
Gm.
60
64
73
73
67
66
67
Curve of Normal Growth ♂
10 cc. orange juice
6338♂
10 cc.
5 cc. orange juice
10 cc. orange juice
6333♂
5 cc. orange juice
10 cc.
5 cc. orange juice
6427♂
10 cc.
5 cc.orange juice
6428♂
10 cc.
5 cc. orange juice
6375♂
15 mg. yeast fraction
5 cc.
2.5 cc. orange juice
6373♂
15 mg. yeast fraction
5 cc.
2.5 cc. orange juice
6372♂
<20 days >
<20 grams>

CHART II.

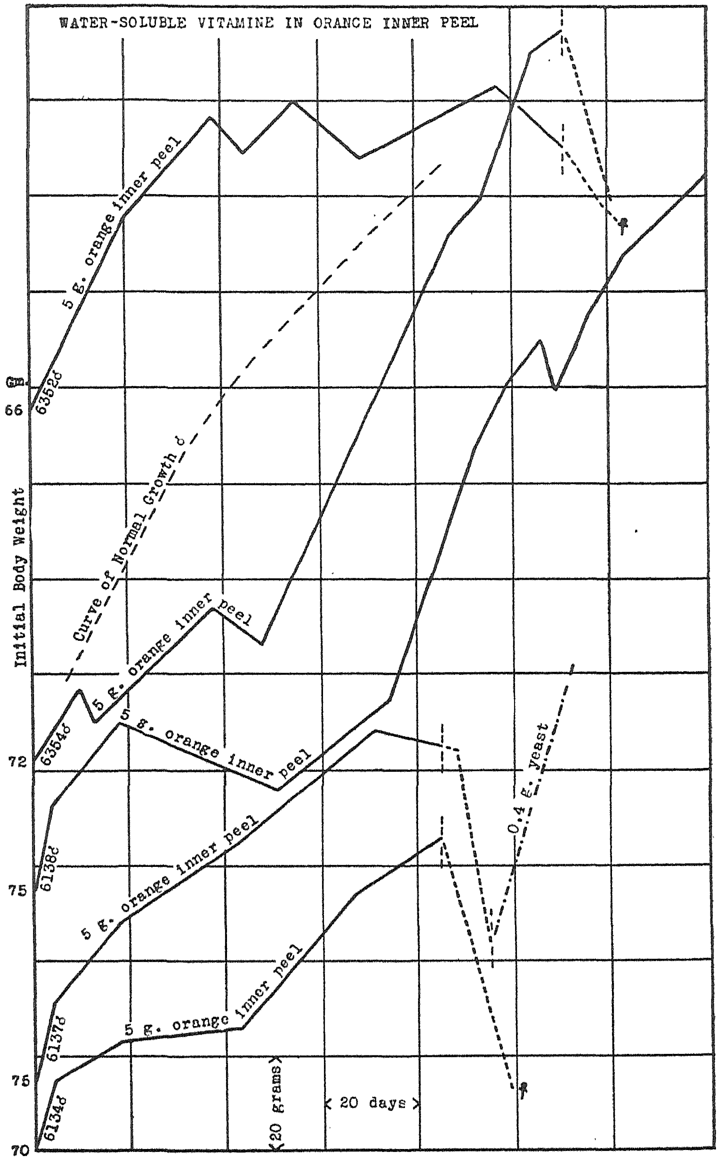
WATER-SOLUBLE VITAMINE IN ORANGE INNER PEEL
5 g. orange inner peel
6352♂
Gm.
66
Curve of Normal Growth ♂
Initial Body Weight
5 g. orange inner peel
6354♂
72
5 g. orange inner peel
6138♂
75
5 g. orange inner peel
6137♂
75
5 g. orange inner peel
6134♂
70
0.4 g. yeast
†
†
20 grams
20 days

CHART III.

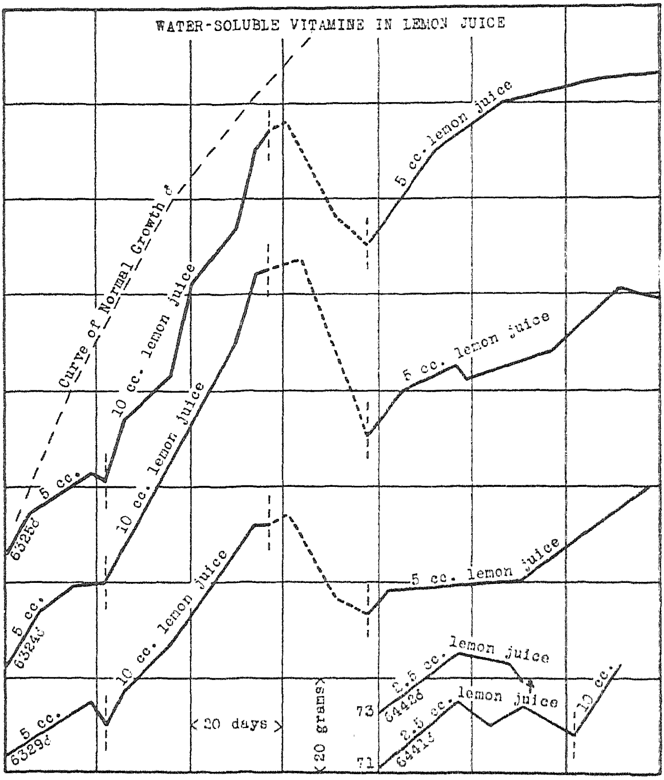
WATER-SOLUBLE VITAMINE IN LEMON JUICE
Initial Body Weight
Gm.
66
63
63
Curve of Normal Growth ♂
5 cc.
6325♂
10 cc. lemon juice
5 cc. lemon juice
5 cc.
6324♂
10 cc. lemon juice
5 cc. lemon juice
5 cc.
6329♂
10 cc. lemon juice
5 cc. lemon juice
< 20 days >
< 20 grams >
73
2.5 cc. lemon juice
6442♂
71
2.5 cc. lemon juice
6441♂
10 cc.

Chart IV.

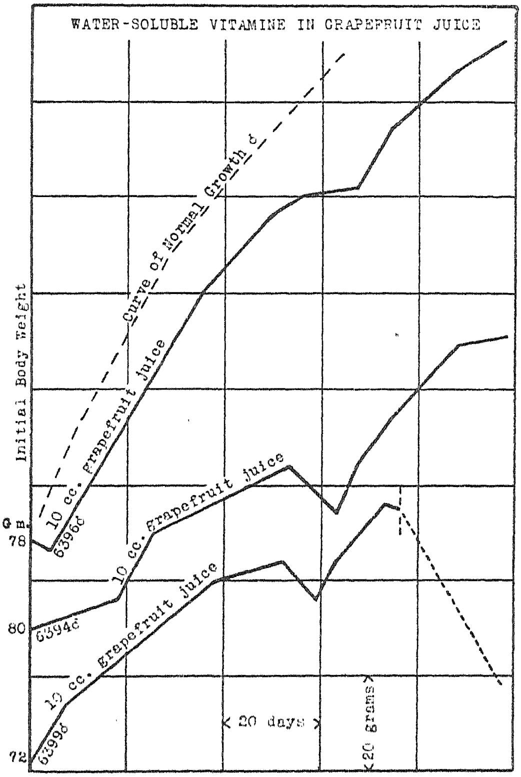
WATER-SOLUBLE VITAMINE IN GRAPEFRUIT JUICE
Curve of Normal Growth ♂
10 cc. grapefruit juice
6396♂
10 cc. grapefruit juice
6394♂
10 cc. grapefruit juice
6399♂
Initial Body Weight
Gm.
78
80
72
< 20 days >
< 20 grams >

CHART V.

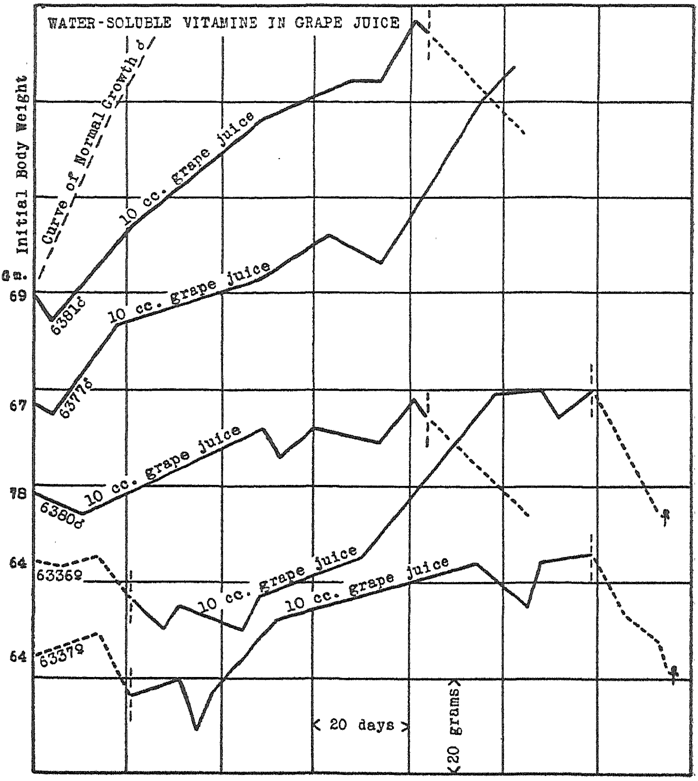
WATER-SOLUBLE VITAMINE IN GRAPE JUICE
Curve of Normal Growth ♂
Initial Body Weight
Gm.
69
67
78
64
64
6381♂
6377♂
6380♂
6336♀
6337♀
10 cc. grape juice
10 cc. grape juice
10 cc. grape juice
10 cc. grape juice
10 cc. grape juice
< 20 days >
<20 grams>

Chart VI.

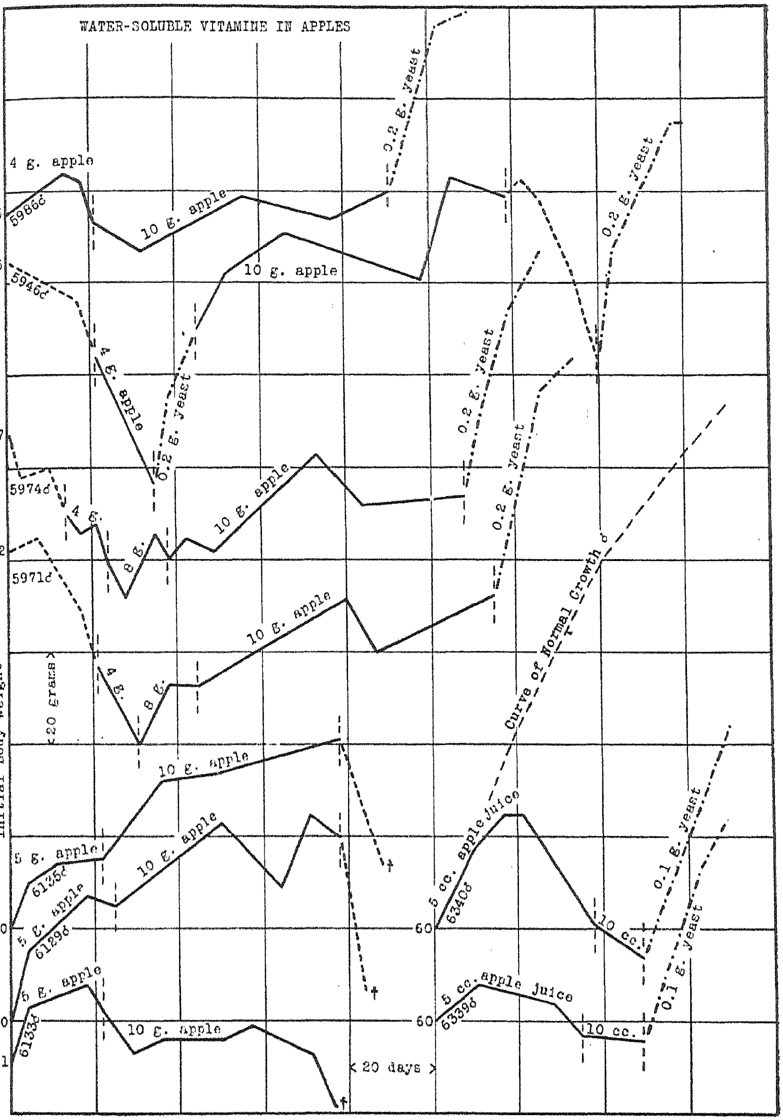
WATER-SOLUBLE VITAMINE IN APPLES
Gm.
145
135
137
132
70
70
71
60
60
Initial Body Weight
<20 grams>
<20 days>
4 g. apple
5986♂
10 g. apple
0.2 g. yeast
0.2 g. yeast
5946♂
4 g. apple
0.2 g. yeast
10 g. apple
0.2 g. yeast
5974♂
4 g.
8 g.
10 g. apple
0.2 g. yeast
5971♂
4 g.
8 g.
10 g. apple
Curve of Normal Growth ♂
10 g. apple
5 g. apple
6135♂
10 g. apple
5 g. apple
6129♂
5 cc. apple juice
6340♂
10 cc.
0.1 g. yeast
0.1 g. yeast
5 g. apple
6133♂
10 g. apple
5 cc. apple juice
6339♂
10 cc.

Chart VII.

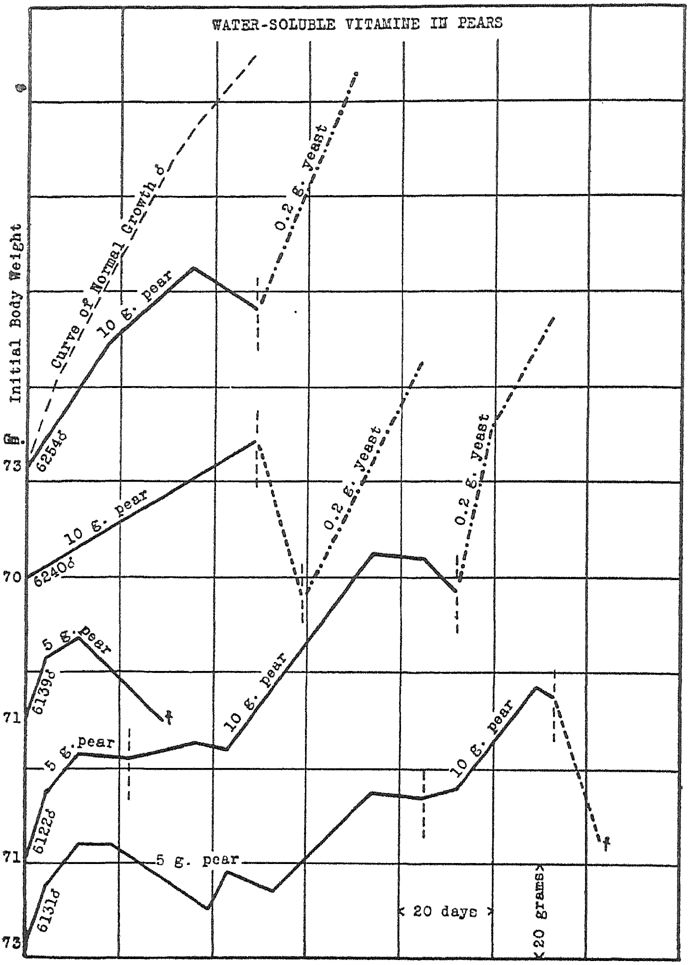
WATER-SOLUBLE VITAMINE IN PEARS
Initial Body Weight
Gm.
73
70
71
71
73
Curve of Normal Growth ♂
10 g. pear
6254♂
0.2 g. yeast
10 g. pear
6240♂
0.2 g. yeast
0.2 g. yeast
5 g. pear
6139♂
†
10 g. pear
5 g. pear
6122♂
10 g. pear
5 g. pear
6131♂
20 days
20 grams
†

Chart VIII.

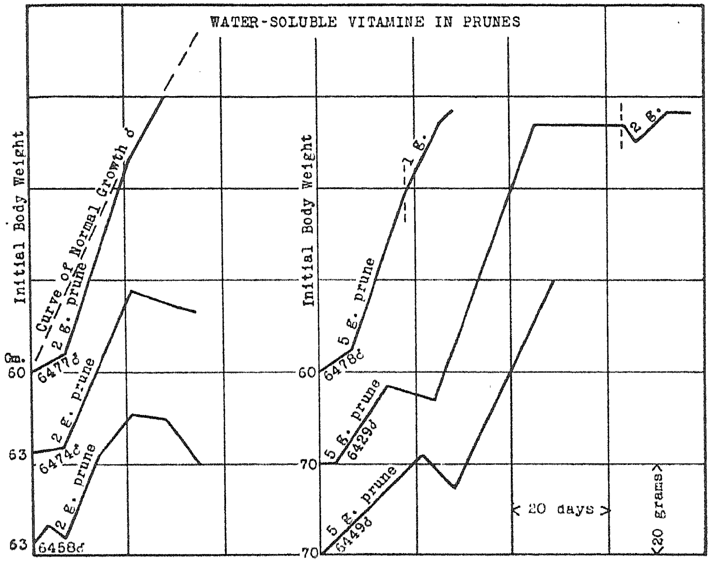
WATER-SOLUBLE VITAMINE IN PRUNES
Initial Body Weight
Gm.
60
63
63
Curve of Normal Growth ♂
2 g. prune
6477♂
2 g. prune
6474♂
2 g. prune
6458♂
Initial Body Weight
60
70
70
5 g. prune
6478♂
1 g.
5 g. prune
6429♂
5 g. prune
6449♂
2 g.
< 20 days >
<20 grams>

CHART IX.

ANTISCORBUTIC PROPERTY OF VEGETABLES.

II. AN EXPERIMENTAL STUDY OF RAW AND DRIED POTATOES.

By MAURICE H. GIVENS and HARRY B. McCLUGAGE.

(*From the Department of Physiology, University of Rochester, Rochester.*)

(Received for publication, May 4, 1920.)

The possibilities of preserving foods by desiccation are becoming recognized more and more. From some points of view drying is undoubtedly preferable to a great many other manipulations of foods to attain this end. However, such procedures should not be adopted without scientific proof that they do not affect adversely the foods dehydrated.

As part of a series of studies on the question whether or not the antiscorbutic vitamine in certain foods is destroyed at definite temperatures during drying we have already reported that under certain conditions cabbage (1), tomatoes (2), and orange juice (3) can be dehydrated so that the resultant dried products retain a significant amount of the antiscurvy vitamine. Data are reported herein concerning the antiscorbutic value of potatoes subjected to various temperatures and treatments.

The white potato is probably our most staple article of diet. Several investigations have been conducted to determine the value of its nitrogen-containing compounds (4-6). Biological experiments are available concerning its content of inorganic salts and water- and fat-soluble vitamines (7). The antiscorbutic property of the potato has been studied somewhat, but we are in need of more information upon this point (1, 8-10).

General Procedure and Methods.

Details of the general procedure and methods are described in the first paper of this series (1). The experimental animals were healthy young guinea pigs. The basal diet used to produce scurvy was made of heated soy bean flour, milk, yeast, paper pulp, calcium lactate, and sodium chloride in the proportions hitherto reported. Tap water was offered to the animals *ad libitum*. Necropsies were performed as soon as possible after death. Scurvy was diagnosed on the basis of the usual clinical manifestations and postmortem findings. The occurrence of pneumonia in some of the animals is ascribed to the possibility that their general resistance was lowered owing to the minimal intake of antiscorbutic vitamine. Almost all the experiments later described are of the preventive type.

Potatoes were fed raw and cooked. The daily allotment of the undried samples was 10 gm. per animal. The dried potatoes or potatoes dried and cooked were fed to each animal in daily quantities of 2.5 gm. On this basis every animal received approximately the same amount of total solids of potatoes equivalent to that contained in 10 gm. of the raw material. When raw potatoes were cooked they were chopped very finely, boiling tap water was poured on them, and the water was kept boiling for 15 minutes. When the dried potatoes were cooked, the powdered material was suspended in a little cold water, poured into boiling water, and the whole boiled for 15 minutes. Potatoes baked and dried were fed as a paste.

Treatment of Potatoes.

In the study of potatoes subjected to different treatments, especial emphasis has been placed on a consideration of dried potatoes. The temperature of drying and the treatment previous to drying have varied. All potatoes dried, with the exception of those baked and dried, were first peeled. The precaution to slice the potatoes and remove the eyes was taken because the nitrogen content of the skin is higher than that of the body of the potato. At present we do not know to what extent the antiscorbutic vitamine may be associated with protein, particularly cellular material. The drying of all samples except Sample F was by means of hot air in a specially constructed apparatus (2).

The different ranges of temperature of drying were 35–40°, 55–60°, 75–80°, and baking at 204°, and then drying at 35–40°C. Potatoes dried at the first three ranges of temperature were

TABLE I.

Sample No.	Date dried.	Date started using.	Date finished using.	Duration of drying.
Potatoes dried at 35–40° C.				
	1918	*1918*	*1918*	*hrs.*
1	July 10	July 12	July 30	6–8
2	" 2	" 31	Aug. 12	6–8
3	" 3	Aug. 13	Sept. 16	6–8
4	" 2	Sept. 17	Oct. 14	6–8
81A	Oct. 10	Oct. 15	Nov. 2	6
81B	" 10	Nov. 3	" 23	6½
Potatoes dried at 55–60°C.				
7	July 10	July 12	July 29	7
16(2)	" 23	" 30	Aug. 12	6¼
19(1)	Aug. 1	Aug. 13	" 26	4
12	July 17	" 27	" 31	6¼
26(1)	Aug. 17	Sept. 1	Oct. 2	4¼
27(1, 2)	" 19	Oct. 3	Nov. 13	4½
Potatoes dried at 75–80°C.				
73(1, 2)	Oct. 7	Oct. 15	Dec. 4	3
			1919	
74(1, 2)	" 7	Dec. 5	Jan. 18	3⅙
	1919	*1919*		
189	Jan. 11	Jan. 19	" 29	2
190	" 11	" 30	Feb. 8	2½
Potatoes baked at 204°C. and dried at 35–40°C.				
	1918	*1918*	*1918*	
109(1, 2)	Oct. 25	Oct. 29	Nov. 26	7¼
			1919	
114(1, 2)	" 31	Nov. 27	Jan. 13	7½
		1919		
148(1)	Nov. 21	Jan. 4	Feb. 5	8½
Potatoes treated with HCl and dried at 55–60°C.				
		1918	*1918*	
67(1, 2)	Oct. 4	Oct. 5	Nov. 6	4¼
Potatoes treated with acetic acid and dried at 55–60°C.				
52(1, 2)	Sept. 21	Sept. 30	Nov. 2	4¼
Potatoes dried *in vacuo* at 55–60°C.				
			1919	
F	Oct. 15	Dec. 23	Feb. 11	10

sliced uniformly with a slicer in the open air and placed in the drier in very thin layers for desiccation. The baked and dried potatoes were placed in an oven at 204°C., kept there for 45 to 55 minutes until they were soft, then removed and scraped out of the shells, put through a ricer, and placed in the drier in thin layers on cheese-cloth to be dried at 35–40°C.

The Sample F potatoes were desiccated in a vacuum drier after they had been sliced under water and dehydrated at a temperature of 55–60°C. for approximately 10 hours under a pressure of less than 2 inches of mercury.[1]

Two lots of potatoes were treated with acid before drying. One lot was sliced and kept in approximately 2 per cent acetic acid for 18 to 20 hours; the other in approximately 0.2 per cent hydrochloric acid for the same length of time. The slices were then taken out of the acid, rolled in towels, and desiccated in the special drier. Two other lots of potatoes were sliced and steamed for 4 minutes before drying at 55–60°C.

All the dried potatoes were gray-colored except those sliced under water and dried in a vacuum, those treated with acid before drying, those baked and dried, and those steamed and dried. This would seem to indicate that enzyme action had been either inhibited or destroyed in these last named products. Details of drying and using are given in Table I.

RESULTS.

It has been demonstrated that a guinea pig fed the basal soy cake diet *alone* will develop clinical manifestations of scurvy in 2 to 3 weeks. Unless at this juncture proper measures are instituted the animal will die in about a week. A critical review of the appended charts shows the following results with differently treated potatoes tested as antiscorbutic supplements to the basal soy cake diet.

In Chart I it is seen that 10 gm. of raw white potato have protected the guinea pigs against scurvy for the duration of the experiment, 129 days—a period in some cases at least six times as long as that in which scurvy would have developed in the absence of antiscorbutic material.

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[1] Our best thanks are due Dr. K. G. Falk for kindly supplying us with this material.

Chart II represents a number of animals which received daily 10 gm. of raw potatoes cooked in water at 100°C. for 15 minutes. There has been no appreciable reduction of the antiscorbutic potency of the potato by this treatment. However, such is not the case if the cooking at 100°C. is continued for 1 hour as shown in Chart IV.

Chart III shows that the antiscorbutic vitamine in raw potatoes cooked for 15 minutes at 100°C. in a weak acid solution, as 0.5 per cent citric acid, is not entirely destroyed by such treatment.

Chart V shows the outcome of feeding daily 2.5 gm. of vacuum-dried potatoes heated in water for 15 minutes at 100°C. The results are not conclusive but indicate that a great part of the antiscorbutic vitamine has been destroyed by this treatment.

Chart VI represents a set of animals to which were fed potatoes dried at 35–40°C. for 6 to 8 hours. Such treatment yields a dried potato which has lost a significant amount of its antiscorbutic potency. A large enough supplement of such potato may afford a slight protection but if the product is heated for 15 minutes in water at 100°C. its value as an antiscorbutic agent is further lessened (Chart VII).

Chart VIII demonstrates that potatoes dried in a blast of air at 55–60°C. for 4 to 6 hours retain some of the antiscorbutic vitamine. However, the quantity of this unknown is further reduced by heating the potatoes in water at 100°C. for 15 minutes (Chart IX).

Charts X and XI show that potatoes dried at 75–80°C. for 2 to 3 hours do not retain so much of the antiscorbutic vitamine as potatoes dried at 55–60°C. for 4 to 6 hours. This statement is based on the fact that several animals showed signs of scurvy on the raw dried potatoes (Chart X) and those guinea pigs represented in Chart XI died in about the usual length of time from the disease. Both of these facts are in striking contrast to the results in Charts VIII and IX.

Chart XII shows that potatoes first heated in the skins at a high temperature and then dried at a low temperature retain enough of the antiscorbutic vitamine in the daily allotment fed to afford the animals protection against scurvy.

Charts XIII and XIV show that treatment with dilute acid (either mineral or organic) before drying does not improve the method of desiccation so far as the antiscorbutic vitamine is concerned.

Chart XV shows the result of feeding potatoes steamed 4 minutes and then dried at 55–60°C. This procedure apparently does not destroy all the antiscorbutic vitamine as some protection was afforded the animals.

Chart XVI shows that potatoes steamed 4 minutes and then dried at 55–60°C. and cooked 15 minutes in boiling water are no longer antiscorbutic agents.

Chart XVII shows that the skins from baked potatoes when dried at 35–40°C. possess no antiscorbutic value.

DISCUSSION.

Attempts have been made by Holst and Frölich (8) and by Chick and Hume (9) to demonstrate experimentally with guinea pigs that raw potatoes contain antiscorbutic material. These investigators failed to succeed because they could not induce the animals to eat the raw food. A little difficulty of this nature was experienced by us with only one animal (Chart I). The reason for our success is attributed to the fact that animals 6 to 8 weeks of age were used and presumably they had not yet developed a discriminative taste. The amount of raw potato fed per guinea pig per day (10 gm.) was the same as has been reported satisfactory for cabbage and tomatoes. That the actual intake of antiscorbutic vitamine was not the same per gm. of substance of each of these foods is very probable if we associate this substance with either the water or total solid content of the foods. The minimal amount of raw potato which will protect a guinea pig against scurvy has not been determined. However, that slightly less than 10 gm. per day is about the lower limit to suffice for protection is indicated from the feeding curve of Animal 204 which from the 35th day to the termination of the experiment, 108 days, consumed on an average about 7 gm. of raw potato daily.

The influence of heat upon the antiscorbutic vitamine appears to be related not only to the degree of temperature but to the duration of the treatment, the reaction, the enzymes present, and the manner of heating.

A temperature of 35–40°C. for 6 to 8 hours seems to be more destructive than 55–60°C. for 4 to 6 hours, and the latter is scarcely as destructive as 75–80°C. for 2 to 3 hours. This conclusion is based on the results obtained with potatoes dried at these temperatures and fed uncooked and cooked for 15 minutes at 100°C. With 2.5 gm. of potatoes dried at 35–40°C. and fed without further treatment, death from scurvy was slightly delayed; with 5 gm. life was certainly prolonged. One out of four animals on a daily dose of 2.5 gm. of potatoes dried at 55–60°C. showed signs of scurvy at death; also one out of three animals on potatoes dried at 75–80°C. showed signs of scurvy at death. If these dried products are further heated in water at a temperature of 100°C. for 15 minutes and then fed to guinea pigs in amounts equivalent to 2.5 gm. of dried material, there is certainly no protection with the 35–40°C. and 75–80°C. products while there is some with the 55–60°C. material.

When potatoes are baked for a short time at a high temperature and then dried, a product is obtained which affords protection against scurvy. Thus it appears possible that the factors involved in the destruction of the antiscorbutic vitamine are not only the degree of heat and the duration of the heating but also the enzyme content and the reaction of the food being dried. Until further light is thrown upon the problem it seems plausible to assume that at any temperature below 80°C. the enzymes are functionating. For example, all potatoes dehydrated below this temperature darkened during the desiccation. Furthermore, samples of these same products tested 9 months after drying showed that they still contained oxidases. The temperature of 35–40°C. is probably the optimum one for the enzyme action; and the duration of drying at this temperature being very long allows several hours for continuous activity. In the case of drying at 55–60°C. and 75–80°C. there is undoubtedly some enzyme action, but the time is reduced. The slight difference in the antiscorbutic value between the products dried at 55–60°C. and at 75–80°C. and then cooked may be accounted for on the basis that at the higher temperature there is more destruction due to the duration of this greater heating and to the reaction. In the case of baking and drying the enzymes are destroyed in a very short time; also the product is subjected to the high temperature for a short period and thus high heat and reaction have little time to act together.

It was thought that some light could be thrown upon the possible effect of enzyme activity and reaction by steaming the potatoes and by treating them with dilute acids before drying. These procedures undoubtedly destroyed the enzymes and permitted a distinct acid reaction during the course of drying, as proved by proper tests. Nevertheless the resultant dried products were unsatisfactory. The above manipulations are open to criticism in several respects: the acid may have extracted most of the antiscorbutic material; or it may have decomposed it; or the combined heat and acid may have destroyed the vitamine.

Holst and Frölich thought that "the active constituent of antiscorbutic food must be of an enzyme nature because exceedingly small amounts of antiscorbutic food exercise such specific action and the substance in some cases is thermolabile." The results with raw potatoes heated at 100°C. and at 204°C. and dried do not support such a contention; they do, however, lend defense to the idea that enzymes under favorable conditions of temperature and reaction may play a rôle in the destruction of the antiscorbutic vitamine.

What is the relation of growth and maintenance to the experimental diet employed and to scurvy? Givens and Cohen have found that their basal diet was perfectly satisfactory to produce normal growth in the rat. It is then logical to assume that the diet contains among its satisfactory components the fat-soluble and water-soluble vitamines. This idea is further supported by the fact that Givens and McClugage were able to maintain pigeons in good health for a very long period on this diet. If this diet is fed alone to guinea pigs scurvy will develop. Growth, as indicated by an increase in weight, will proceed for a short time during the development of scurvy and is arrested only when the disease has progressed to a marked stage. Maintenance can be secured for only a few days during the rapid development of the malnutrition. A decline sets in very shortly and death soon follows unless a suitable addition of antiscorbutic vitamine to the dietary is made.

The experiments herein reported lend striking confirmation to the belief that there is a third type of vitamine—one protecting against scurvy. Osborne and Mendel (11) have found from long experience that 0.2 gm. of dried brewer's yeast per rat per day is sufficient to promote normal growth. The basal diet used for producing scurvy contained more than twice that amount. There is no basis to warrant the allegation that the drying of the basal diet or potatoes has destroyed either the fat- or water-soluble vitamine. Chick and Hume (9) and more recently Daniels and McClurg (12) have shown that the water-soluble vitamine is not destroyed by long exposure to a temperature of 100°C. Furthermore Osborne and Mendel (13) have reported that drying spinach at 55–60°C. does not apparently destroy either of the above named factors.

In an interesting study of the dietary properties of the potato McCollum and associates (7) have demonstrated that this food is deficient in calcium, sodium, chlorine, the quality of protein, and the fat-soluble vitamine. All these limiting factors have been supplied in the basal diet used in our experiments.

Holst and Frölich were unable to induce guinea pigs to eat either fresh uncooked or dried uncooked potatoes. They therefore resorted to the use of cooked potatoes. The cooking was for approximately $\frac{1}{2}$ hour in salt water. As dried products they used commercially dried potatoes (concerning which no details are given other than the potatoes were first soaked in dilute sulfuric or hydrochloric acid before dehydrating), potatoes dried in the air at 37°C., and some dried *in vacuo* at 30°C. The animals received no other food except potatoes. With fresh cooked potatoes given *ad libitum* there was apparently almost complete protection from scurvy up to 127 days. However, death ensued sooner or later and there was a marked drop in weight of all animals. This can be explained now in the light of McCollum's experiments with rats whereby it has been determined that the potato is deficient in several respects. Holst and Frölich found with potatoes commercially dried, air-dried at 37°C., and vacuum-dried at 30°C. and then cooked that there was no protection against scurvy.

Our results with fresh potatoes cooked and with potatoes dried at 35–40°C. and cooked confirm those of Holst and Frölich. With fresh potatoes cooked the findings herein reported are in accord with the experience of Chick and Hume.

1. Givens, M
2. Givens, M.
3. Givens, M.
30.
4. Hindhed
Am. M.
5. Thor
6. F

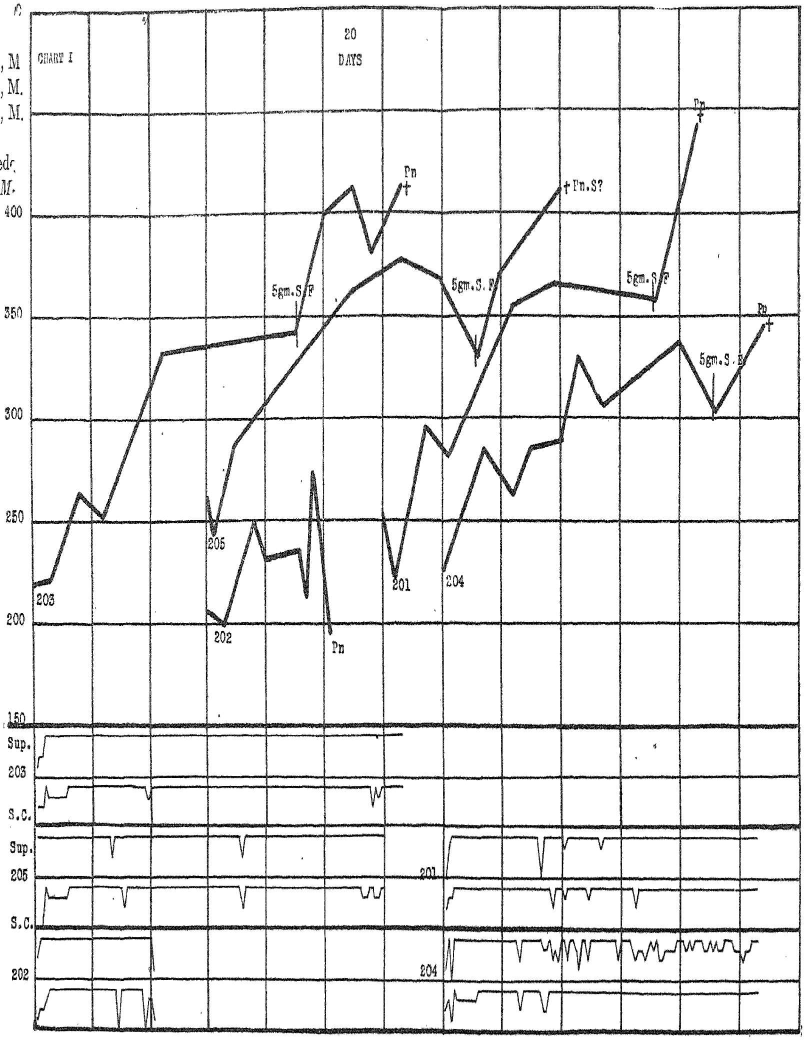

CHART I. This chart shows that a small daily allotment of raw potatoes will prevent the occurrence of scurvy in guinea pigs. 10 gm. of raw potatoes were consumed by each animal except No. 204 whose average intake was about 7 gm. However, this lessened intake did prevent the appearance of any external scorbutic manifestations within the experimental period. Pneumonia accounted for the death of all animals in this group. In the case of No. 205 death was undoubtedly due to pneumonia but whether it was complicated by scurvy is a question. At autopsy this animal showed a few subcutaneous hemorrhages. As the animals increased in weight and age more food was needed as is shown by the way all of them responded to a daily addition of 5 gm. of soy bean flour heated for 30 minutes at 20 pounds pressure.

5 gm. S F = 5 gm. of heated soy bean flour added to the diet.

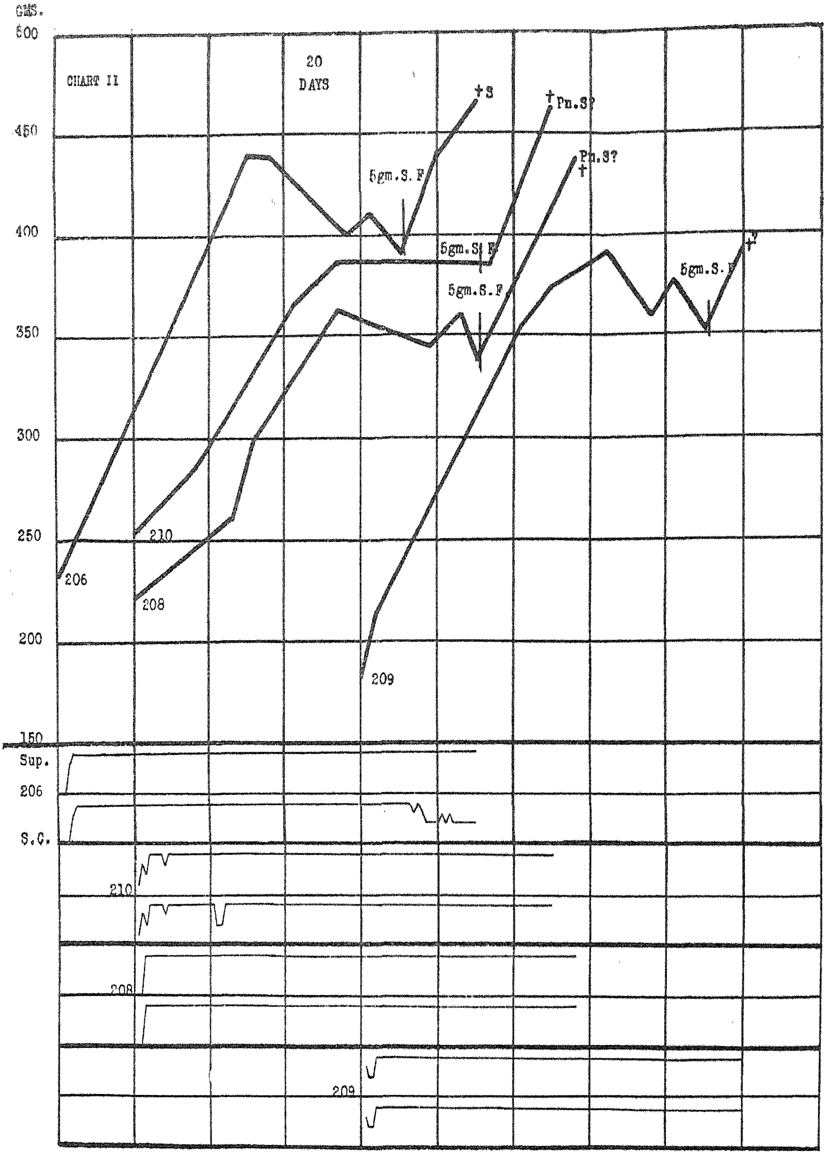

CHART II. These curves show that a daily allotment of 10 gm. of raw potatoes cooked for 15 minutes will protect guinea pigs against scurvy for a very long period. Most of these animals lived as long as those on raw potatoes, in Chart I, but one guinea pig (No. 206) had definite signs and two others questionable evidences of scurvy at death. It therefore seems safe to conclude that even heating the potato at 100°C. for 15 minutes does slightly diminish the content of antiscorbutic vitamine.

5 gm. S F = 5 gm. of heated soy bean flour added to the diet.

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Key to Charts.

Sup. = Supplemental intake.
S. C = Basal soy cake diet.
Pn. = Pneumonia.
S = Scurvy.
S? = Scurvy questionable.
Pn.S? = Pneumonia with questionable scurvy.
Pn I. = Pneumonia and impaction of the intestines.
I = Impaction of the intestines, cecum.
? = Cause of death a question.
OK = Animal apparently recovered.
* = Termination of the experiment.
† = Death.

The growth curves, which are self-explanatory, are separated from the feeding curves by a large heavy base line which extends out to the left under the weight figures. The feeding curves, always at the bottom of the chart, are plotted on a basis of 4. In these curves there are five equal subdivisions between the base lines. Consequently if an animal were eating all his food the curves of supplemental and basal intakes would be parallel to the base lines four-fifths of the distance above them. The number of the animal to which the feeding curves belong is always placed on the left opposite the base line separating the curve of supplemental intake (Sup.) from that of the intake of the basal diet (S. C). The curve of the supplemental intake is always above that of the basal food intake. The time relation is the same for all curves; that is, 20 days to each square.

As an example of the food curves, take No. 203, Chart I. On the 1st day this animal ate one-fourth his supplemental intake, on the 2nd and 3rd days one-half, and on the 4th day and every day thereafter he ate all of it. The basal diet curves (S. C) are read in the same way depending upon the depth of the break on the basis of 4.

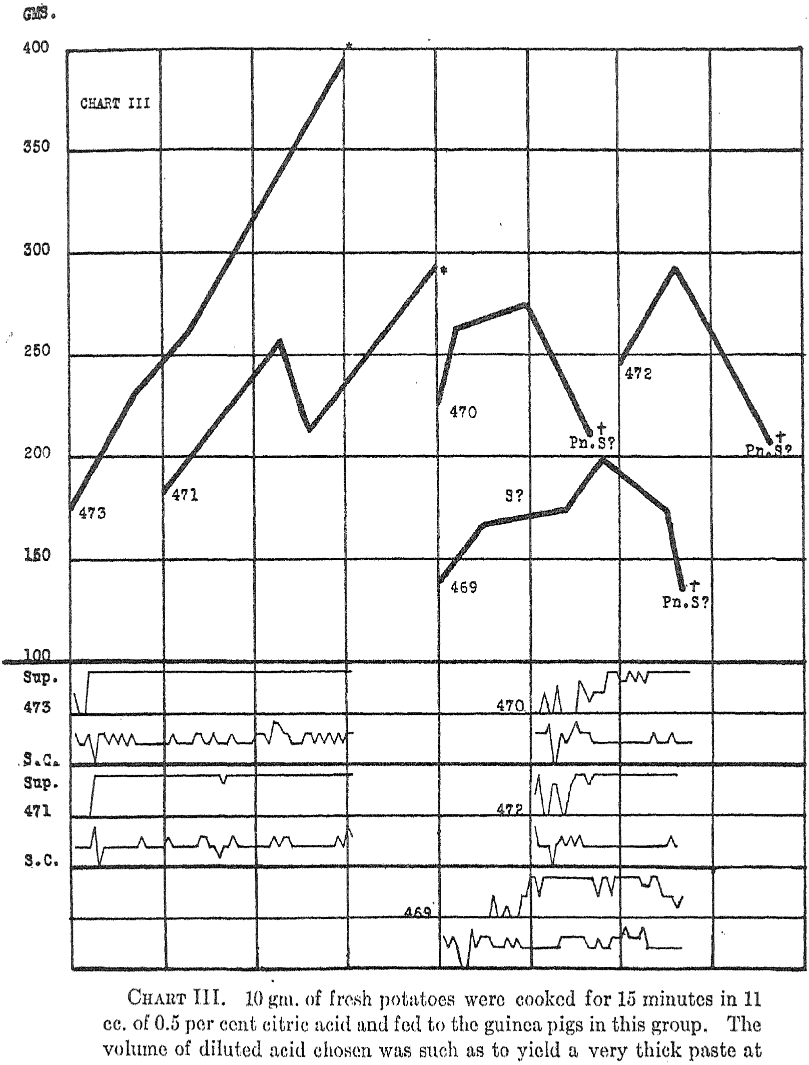

Chart III. 10 gm. of fresh potatoes were cooked for 15 minutes in 11 cc. of 0.5 per cent citric acid and fed to the guinea pigs in this group. The volume of diluted acid chosen was such as to yield a very thick paste at the end of the cooking time. Unfortunately only two of the animals in this group consumed the entire daily supplement of potatoes and the experiments had to be terminated at 60 days for business reasons. However, the indications are that the amount of citric acid used was not destructive and it may have been beneficial. That the latter may be the case when the cooking is for 1 hour is noted in Chart IV.

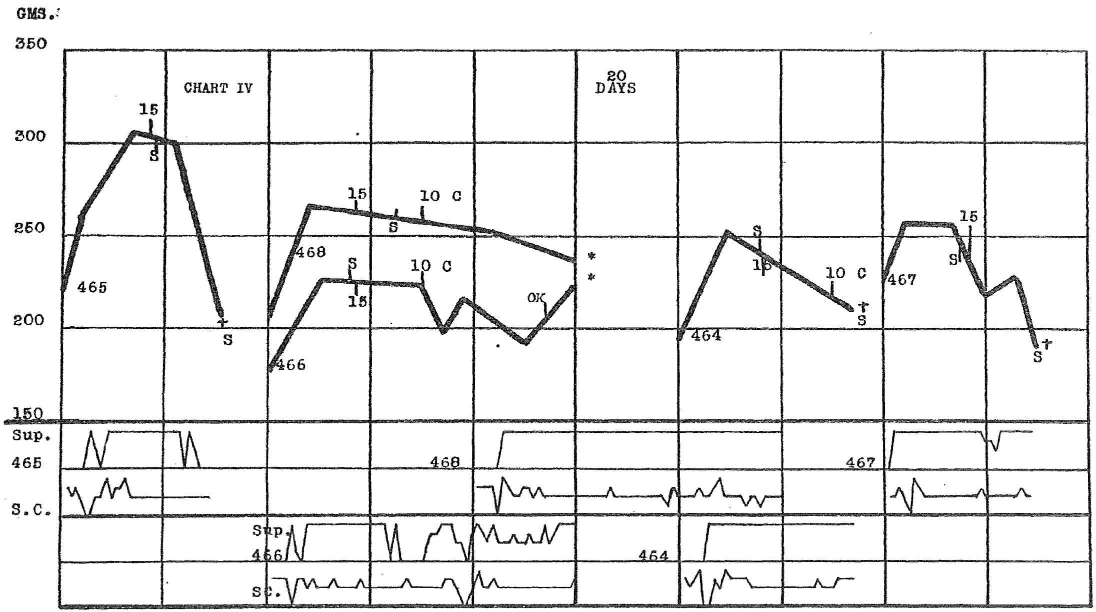

CHART IV. These curves show that 10 gm. of potatoes cooked for 1 hour at 100°C. have their vitamine content so much reduced that scurvy is not prevented. The disease could not be arrested by feeding 15 gm. of potatoes so cooked. In the case of Nos. 466 and 468 scurvy was checked by feeding 10 gm. of potatoes cooked in 11 cc. of 0.5 per cent citric acid for 1 hour. This would seem to indicate that a slight organic acidity may prevent some of the deleterious action of heat upon the antiscorbutic vitamine.

15 = 15 gm. of potatoes.
10 C = 10 gm. of potatoes cooked in 11 cc. of 0.5 per cent citric acid 1 hour.

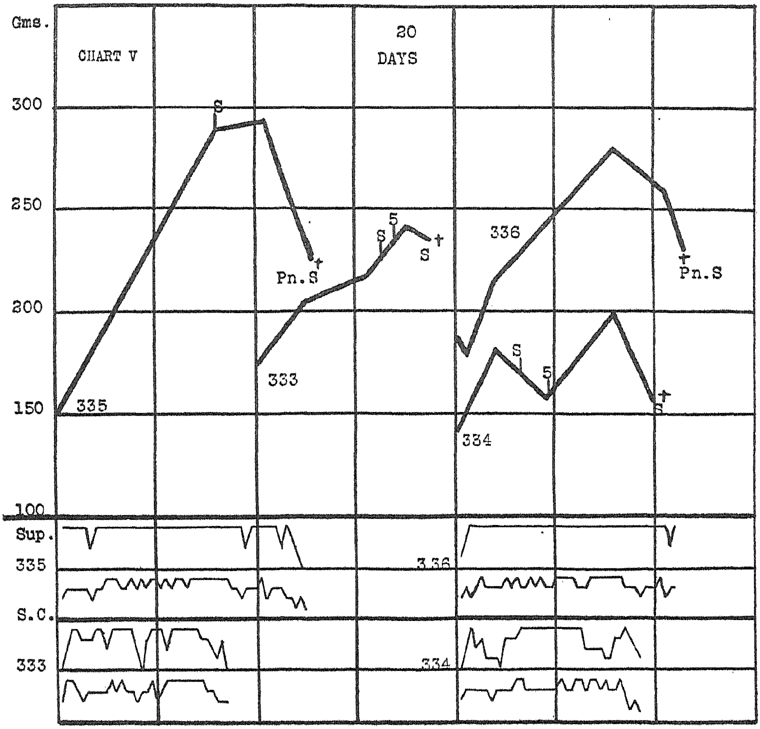

Chart V. These curves represent the result of feeding potatoes dehydrated at a low temperature *in vacuo*. This mode of desiccating potatoes yields a product which is not entirely devoid of some antiscorbutic property if one can judge by the fact that the onset of scurvy has been delayed. 5 = 5 gm. or a double dose of potatoes fed.

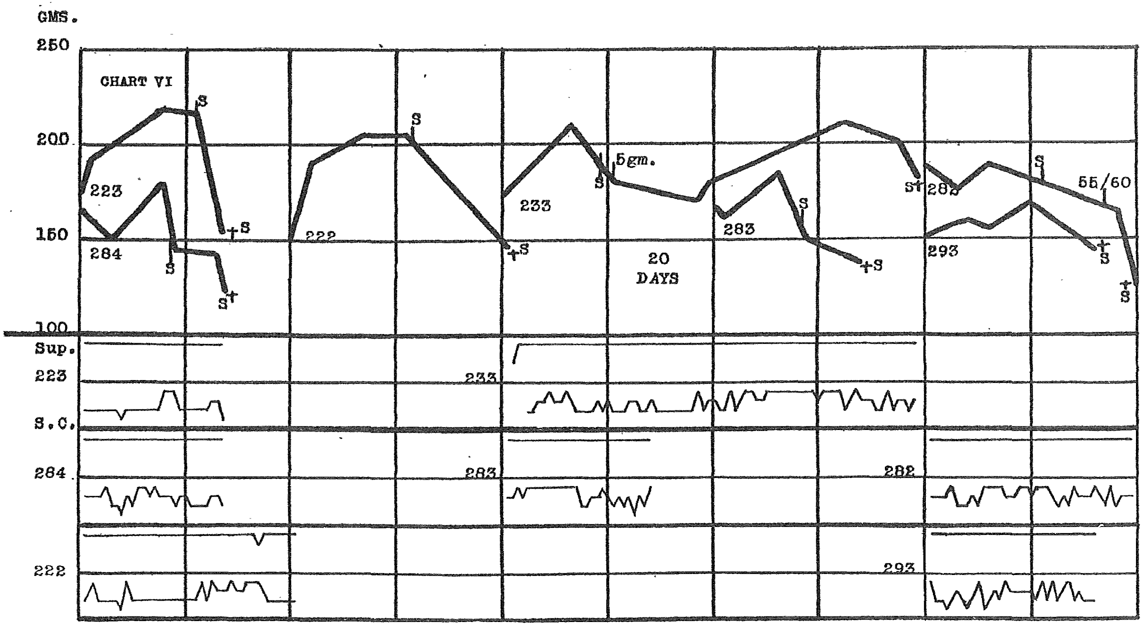

Chart VI. The animals in this group were fed potatoes which had been dehydrated at 35–40°C. Such a product contains practically no antiscorbutic potency. That it is not entirely free of the antiscorbutic vitamine is shown in the case of No. 233 which was fed 5 gm. of the dried potatoes daily after the appearance of scurvy. This animal was maintained for 60 days after the appearance of the disease but finally succumbed regardless of the double daily dose of dried potatoes.

5 gm. = 5 gm. or a double dose of potatoes fed.

55/60 = Potatoes dried at 55–60°C. substituted for those dried at 35–40°C.

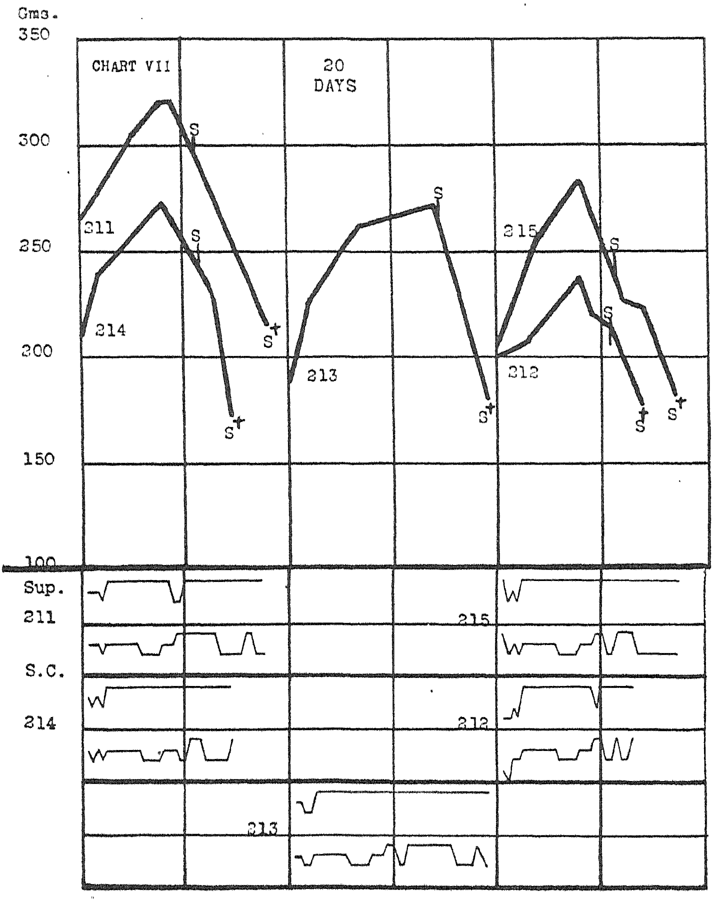

Chart VII. These curves show that potatoes dehydrated at 35–40°C. and then cooked for 15 minutes at 100°C. afford no protection against scurvy. The amount here fed corresponds to 10 gm. of raw potatoes which, as is seen from Chart I, will protect guinea pigs against the disease.

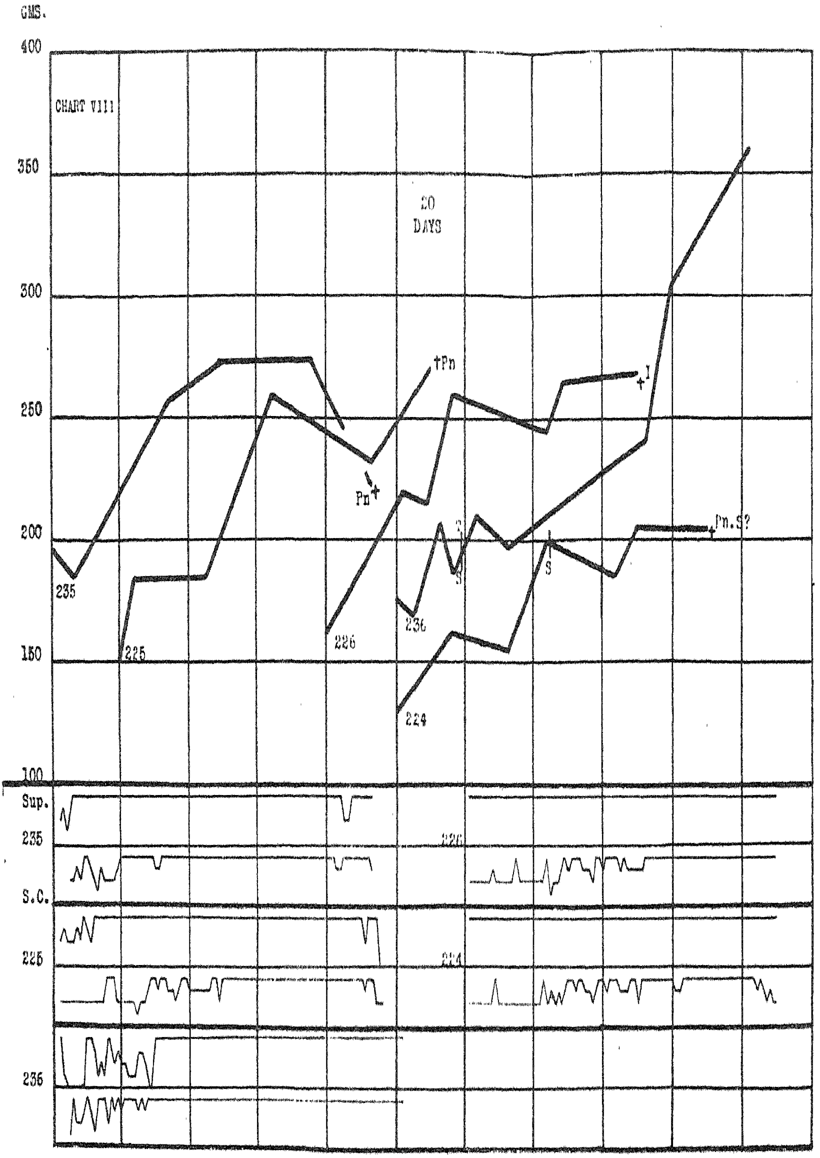
GMS.
400
350
300
250
200
150
100
CHART VIII
20 DAYS
†Pn
Pn†
†I
†Pn.S?
S
S
235
225
226
236
224
Sup.
235
S.C.
225
236
226
224

CHART VIII. These curves demonstrate that potatoes dehydrated at 55–60°C. contain enough of the antiscorbutic essential to prevent the development of scurvy over a period three times as long as that in which the disease would appear if no protection were afforded. Three of the animals (Nos. 235, 225, 226) showed no signs of scurvy at the time of death (90 days after beginning the experiment); succumbing in the case of the first two to pneumonia and the other to an apparent impaction. No. 236 would not eat the potatoes and accordingly developed scurvy; with 10 gm. of fresh tomatoes cooked 15 minutes at 100°C. it was cured and carried for over 100 days when removed from the experiment. No. 224 showed some external indications of scurvy, as tender joints, at 45 days; however, it shortly improved and at death, 45 days later, showed questionable signs of scurvy.

T = Tomatoes introduced into the diet.

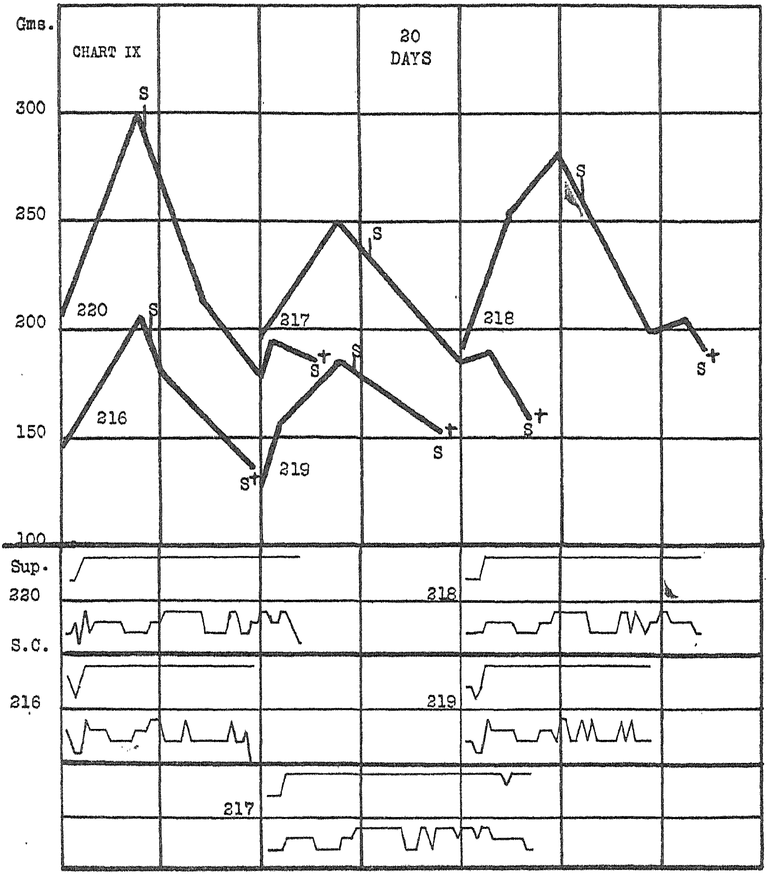

Chart IX. Potatoes dehydrated at 55–60°C. and then cooked for 15 minutes at 100°C. were fed the guinea pigs represented by the curves. The daily supplement was 2.5 gm. of dried material. None of the animals was protected against scurvy. Such antiscorbutic potency as may remain after dehydration at 55–60°C. is apparently rendered *nil* by the short cooking process at 100°C.

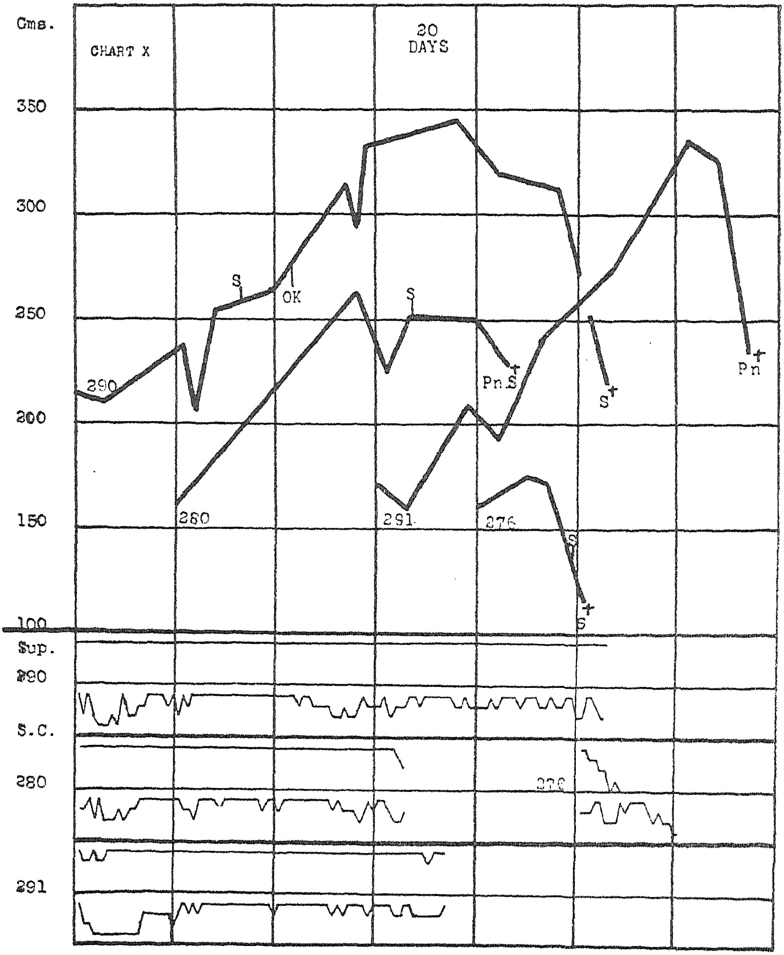

Chart X. 2.5 gm. of potatoes dehydrated at 75–80°C. were fed as a daily allotment to this group of animals. Such a product undoubtedly contains some of the antiscorbutic vitamine but the amount is certainly not equivalent to that contained in potatoes dried at 55–60°C.

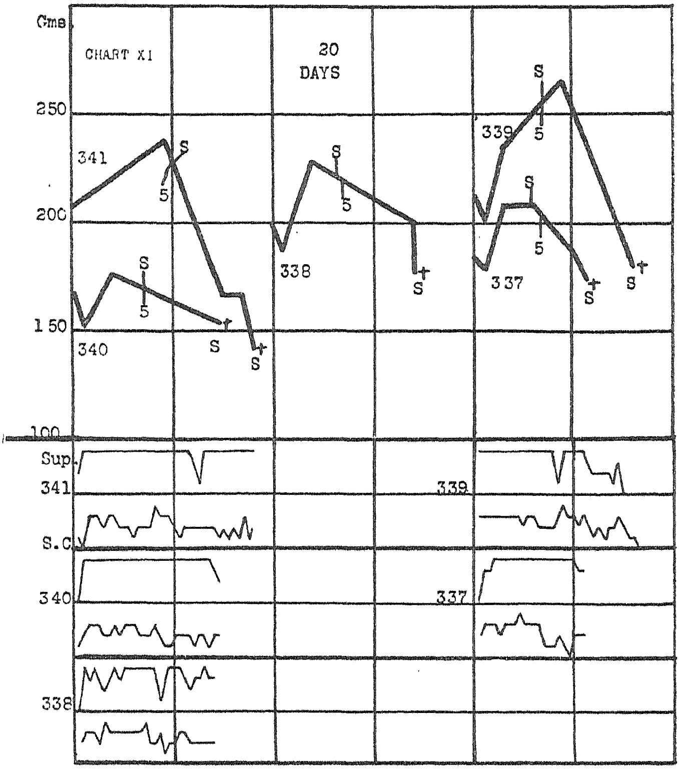

Chart XI. 2.5 gm. of potatoes dried at 75–80°C. were cooked for 15 minutes at 100°C. and fed to this group of guinea pigs. No protection against scurvy was afforded the animals even when the dosage was doubled. Again the destructive effect of cooking at 100°C. on the antiscorbutic vitamine is shown.

5 = 5 gm. or a double dose of potatoes fed.

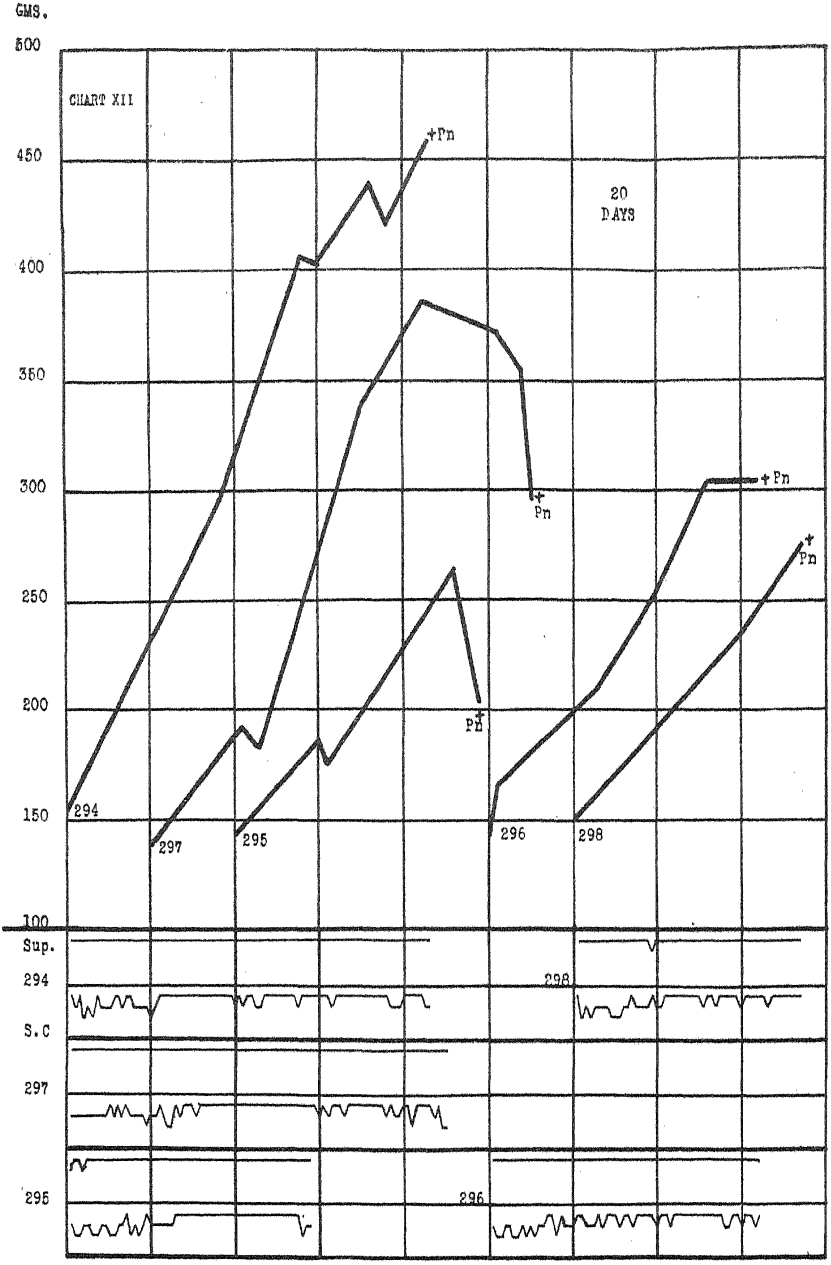
GMS.
500
450
400
350
300
250
200
150
100
CHART XII
20
DAYS
+Pn
+Pn
+Pn
+Pn
+Pn
294
297
295
296
298
Sup.
294
S.C
297
295
298
296

Chart XII. Potatoes were baked in the skins for 45 to 55 minutes at 204°C., then scooped out, and dried at 35–40°C. This dried product was fed in 2.5 gm. doses daily and protected the guinea pigs against scurvy. All the animals died from pneumonia showing none of the lesions of scurvy. It seems indicated from these experiments that subjection for a short time to a high temperature and then drying at a low one is less destructive than drying at a low temperature alone.

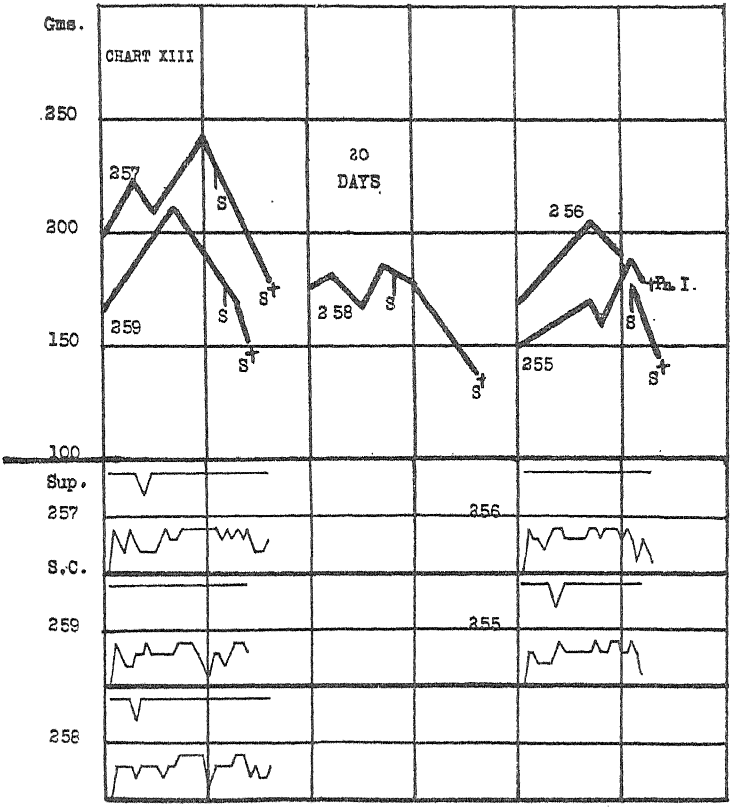

CHART XIII. Potatoes were soaked over night in dilute hydrochloric acid, dried at 55–60°C., and then cooked for 15 minutes at 100°C 2.5 gm. of dried material so treated afforded no protection against scurvy. In previous experiments (Chart IX) it is seen that the animals lived longer on the potatoes dried at 55–60°C. and cooked 15 minutes than the animals represented by the above curves. The mineral acid in connection with the heat employed or the extraction of the vitamine by the acid treatment or both factors combined lessened the antiscorbutic potency of the product.

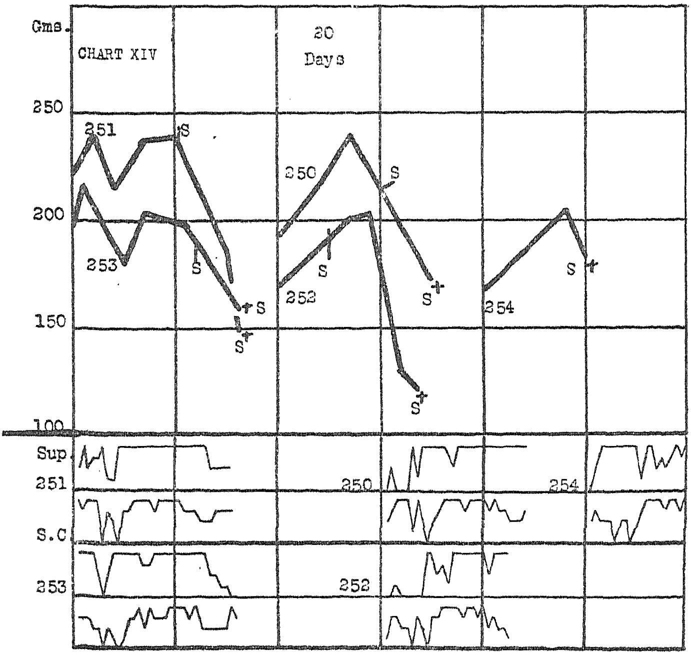

CHART XIV. Potatoes soaked over night in dilute acetic acid, dried at 55–60°C., and then cooked for 15 minutes at 100°C. were fed to guinea pigs in this group. This product was ineffective in preventing scurvy.

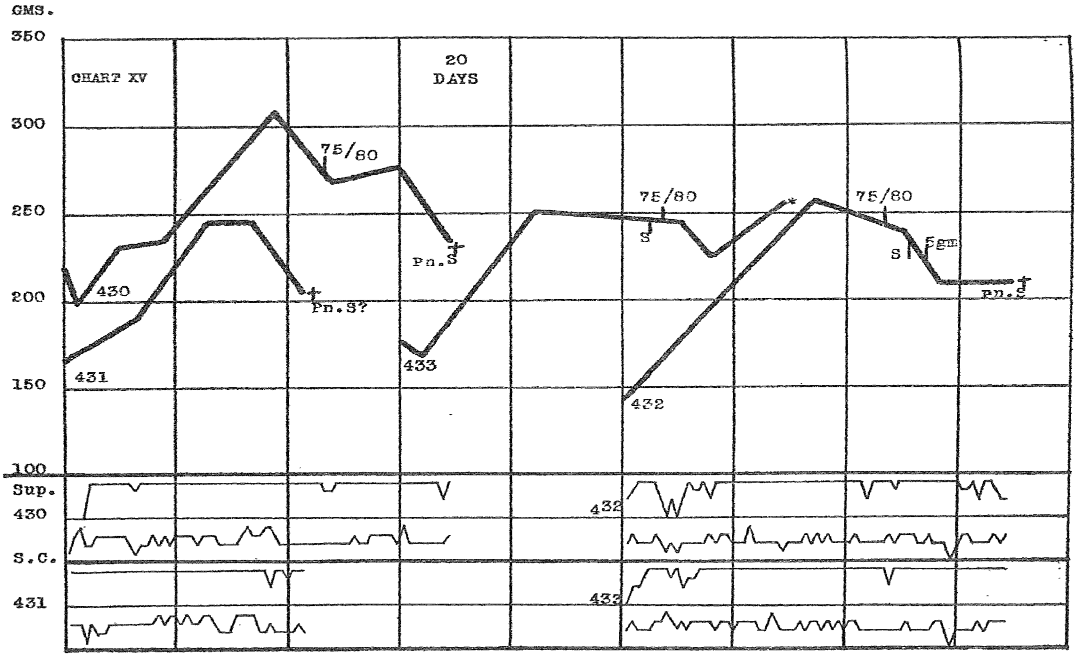

CHART XV. Potatoes steamed for 4 minutes and then dried at 55–60°C. were fed to the guinea pigs represented by the above curves. One animal died at 43 days from pneumonia showing signs of scurvy. Accordingly all animals were transferred to 2.5 gm. of potatoes dried at 75–80°C. Two of them died from pneumonia showing scorbutic symptoms; the other one was apparently improved at 70 days when the experiment had to be terminated.

75/80 = Potatoes dried at 75–80°C. substituted for those dried at 55–60°C. after a previous steaming for 4 minutes.

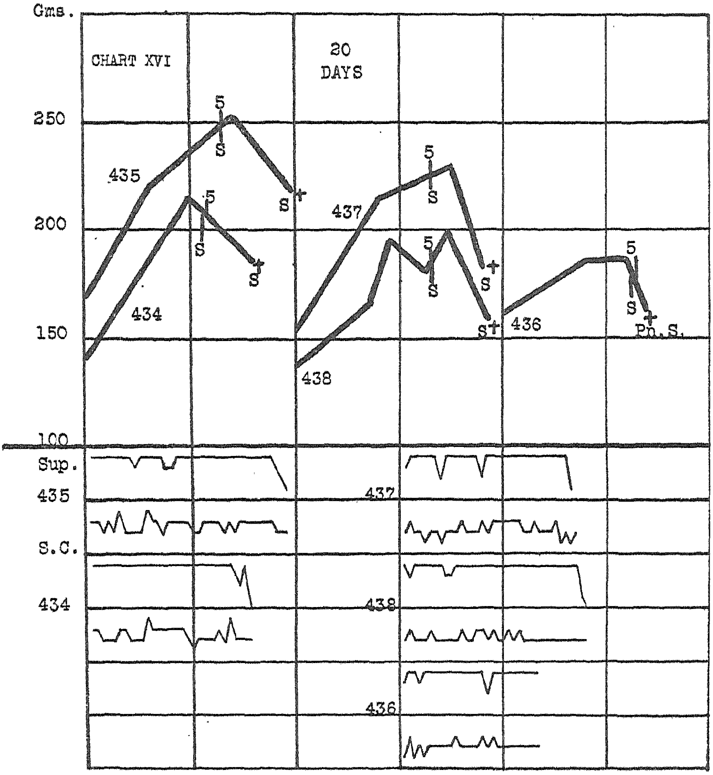

Chart XVI. Potatoes were steamed for 4 minutes, dried at 55–60°C., and cooked for 15 minutes at 100°C. A daily dose equivalent to 2.5 gm. of dried material failed to afford these animals any protection against scurvy, even when the dose was doubled.

5 = 5 gm. or a double dose of potatoes fed.

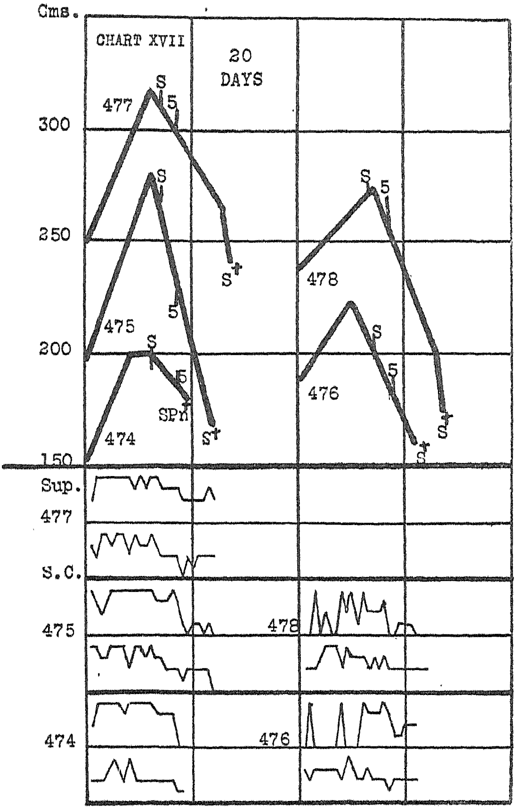

CHART XVII. The potato shells from the baked potatoes were dried at 35–40°C. and 2.5 gm. fed daily. This material even in a double dose failed to protect the animals against scurvy.

5 = 5 gm. or a double dose of potato shells fed.

NEUTRALITY REGULATION IN CATTLE.*

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Subsequent to the publication of a method for the determination of the carbon dioxide-combining power of blood plasma by Van Slyke (1917), much of value has been contributed to our knowledge of the alkaline reserve of the animal body. Normal values and the changes produced by various pathological and experimental conditions have been established for a number of mammals. Naturally, the changes occurring in man have been most studied, and the amount of carbon dioxide which the plasma is able to hold in combination as bicarbonate is justly regarded as a convenient and accurate measurement of the alkaline reserve of the body.

As far as I have learned, no one has attempted a study of the alkaline reserve of cattle. In the course of an investigation of the physiology of milk secretion in progress in this laboratory, it became of great importance to discover the changes occurring in the acid-base balance of cattle, and to attempt to gain an insight into the manner in which these animals maintain their tissue neutrality.

It has long been a well known fact that cows normally excrete a strongly alkaline urine which is loaded with carbonate, as shown by the violent ebullition of carbon dioxide when an acid is added. This strongly alkaline reaction is justly referred to the diet of cows, one in which there is commonly a large excess of basic elements. On the other hand, calves which consume an approximately neutral diet, milk, commonly excrete urines that are neutral or amphoteric to litmus. Judging from these facts one would expect calves to have a smaller alkaline reserve than cows. In-

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deed there are many observations on man that lend support to this hypothesis. Marriott (1916, *a*) has shown that the blood of infants has a higher "fixed acidity" than that of adults, and that the alveolar CO_2 tension of infants is low (1916, *b*). Children are known to be especially susceptible to acidotic conditions.

Meigs, Blatherwick, and Cary (1919, *a*) have pointed out that the plasma of young calves contains more calcium than that of cows, and have suggested that this might be connected with a lower CO_2 capacity in the case of calves. Rona and Takahashi (1913) studied the solubility of calcium in solutions of $NaHCO_3$ and showed that if the concentration of hydrogen ions remains constant the amount of calcium in solution varies inversely with the bicarbonate. This taken with the observations of Allers and Bondi (1907), who were able to double the concentration of the blood calcium by feeding hydrochloric acid to rabbits, made it desirable to determine the concentration of calcium and the carbon dioxide-combining power of plasma under various conditions. In several instances, estimations of the phosphorus content of plasma, and of the urinary excretion of calcium, phosphorus, ammonia, and carbon dioxide have been made.

Methods.

Carbon dioxide-combining power of plasma was determined by the well known method of Van Slyke (1917), calcium and phosphorus by the methods described by Meigs, Blatherwick, and Cary (1919, *a*, *b*), and ammonia in the urine by Steel's (1910) modification of the Folin procedure. Estimations of the carbon dioxide content of urine were made in the Van Slyke apparatus. Many urines contain so much carbonate that a 1:4 dilution must be made and the determination carried out on this solution.

Blood samples were obtained at about 9 a.m., approximately 4 to 5 hours after the last feeding. Blood was taken from the jugular vein through a large cannula. When blood is allowed to flow rapidly through a large cannula in this manner and is immediately centrifuged there is no appreciable loss of carbon dioxide. I have several times made check determinations with samples taken under paraffin oil and am fully convinced of this fact. Plasma from blood to which saturated sodium citrate solution is added in 1 per cent concentration to prevent coagulation also gives the same values for CO_2 capacity, as does the corresponding plasma in which sodium oxalate is used as an anticoagulant.

On account of the inherent difficulties of obtaining 24 hour specimens of urine from cows, I have collected the first urine voided after the blood sample was secured. It is realized that the values from single specimens may vary somewhat from those obtaining for the urine of a complete day. However, it is thought that such results represent actual conditions closely enough for the purposes in view.

Carbon Dioxide Capacity of Cow Plasma.

Examination of the values shown in Table I reveals the remarkable constancy of the alkaline reserve of the cow. Twenty-two determinations of the CO_2 capacity of sixteen individuals gave an average value of 61.5 cc. of CO_2 bound by 100 cc. of plasma. The maximum and minimum figures were 68.3 and 55.1 cc., respectively. The table is arranged beginning with the lowest CO_2 capacity and extending to the highest, except when more than one determination has been made at a different date on an animal, in which case the values follow chronologically. No evidence of the acidosis of pregnancy observed in women by Hasselbalch and Gammeltoft (1915), and by Losee and Van Slyke (1917) is indicated by the CO_2-combining power of the plasma of pregnant cows. In fact, the majority of pregnant animals gave values about or higher than the average; *i.e.*, a greater alkaline reserve. None of the figures here given can be interpreted as showing the presence of an acidotic condition, and it may be justly said that the determination of plasma CO_2 capacity of cows gives no indication of slight tendencies in this direction. The variations in the alkaline reserve are such as can be duplicated by appropriate changes in rations, as will be demonstrated in another part of this paper.

TABLE I.

Carbon Dioxide-Combining Power of Cow Plasma.

Date.	Cow No.	CO_2 capacity per 100 cc.	Remarks.
		cc.	
Aug. 11, 1919	121	58.9	Farrow.
Oct. 9, 1919	121	55.1	“
June 26, 1919	119	56.0	No morning feed. Pregnant, 7th month.
Sept. 8, 1919	84	56.9	No morning feed. Farrow.
Nov. 13, 1919	84	57.9	CO_2 in urine, 247 cc. per 100 cc. Farrow.
Jan. 14, 1920	84	63.6	Farrow.
Sept. 15, 1919	89	58.9	Pregnant, 3rd month.
Jan. 7, 1920	89	64.0	“ 7th “
Feb. 17, 1920	63	59.5	Not lactating.
Sept. 10, 1919	88	59.7	Pregnant.
Nov. 18, 1919	88	62.6	CO_2 in urine, 235 cc. per 100 cc. Pregnant.
Feb. 25, 1920	88	61.7	Pregnant. Due to calve March 19.
“ 13, 1920	221	60.3	Pregnant, 8th month.
Aug. 22, 1919	114	60.6	Aborted 2 days before; 5th month of pregnancy.
“ 19, 1919	220	62.6	Farrow.
“ 27, 1919	249	63.1	Calved 10 days later.
Oct. 15, 1919	64	64.5	Milking.
June 19, 1919	82	64.5	Farrow.
Aug. 26, 1919	244	64.5	Calved 1 day later.
Dec. 3, 1919	56	64.6	Farrow.
Nov. 28, 1919	51	65.5	Milking.
Jan. 15, 1920	49	68.3	Aborted preceding day; about 5 weeks before time.

The Alkaline Reserve of Calves.

Table II gives among other things the amount of CO_2 chemically bound by the plasma of young calves. At a glance one sees that calves possess a larger alkaline reserve than do grown cattle. The average CO_2 capacity of the plasma of seven calves ranging in age from 2 to 14 days is 73.0 cc., with maximum and minimum values of 80.6 and 68.3 cc., respectively. Calf 309, when it was slightly over 1 month old, showed a CO_2 capacity of 61.4 cc., practically identical with the average adult figure.

Attention is also invited to the values obtained for urinary carbon dioxide. Calves excrete approximately neutral urines containing from 3 to 17 cc. of CO_2 per 100 cc., while the strongly alkaline urine of cows usually contains from 200 to 400 cc. of CO_2 in 100 cc. But in spite of this calves have a decidedly greater CO_2 capacity, amounting on the average to a difference of 11.5 cc. per 100 cc. of plasma. It is evident that a fundamentally different regulatory mechanism is functioning in the two cases.

TABLE II.

Composition of Plasma and Urine of Young Calves.

Date.	Calf No.	Age.	Plasma.			Urine.			Remarks.
			CO_2 capacity per 100 cc.	Ca per 100 cc.	P (inorganic) per 100 cc.	Specific gravity.	CO_2 per 100 cc.	NH_3 N per 100 cc.	
1919		*days*	*cc.*	*mg.*	*mg.*		*cc.*	*mg.*	
Sept. 27	309	33	61.4	11.1	7.5	1.006	5.7	17.0	
Aug. 29	309	2	68.3	12.1					Blood obtained after some struggling.
1920									
Feb. 5	314	4	70.2	10.6	6.8	1.015	17.3	9.7	
" 16	310	36	71.0	10.5	7.7	1.007	9.7	14.6	P in urine, 47.3 mg. per 100 cc.
Jan. 13	310	2	74.8	13.2	6.8	1.006	14.0	8.3	
Feb. 9	312	10	71.1	12.4	8.2	1.016	8.8		
" 10	311	14	73.0	11.5	9.3	1.021	5.0		
" 12	315	4	73.0	12.2	6.7	1.006	2.9		
" 7	313	7	80.6	12.3	7.3	1.004	13.3		P in urine, 30.8 mg. per 100 cc.

The values in Table II corroborate and extend the observations of Meigs, Blatherwick, and Cary (1919, *a*), who showed that the plasma of calves is characterized by a high content of calcium and inorganic phosphorus. As was pointed out in that communication, calcium soon begins to decrease toward the adult level but the phosphate gains in amount until the highest point is reached at an age of about 6 months.

The urine of young animals as compared with that of cows contains less carbon dioxide, more ammonia, and more phosphorus. Many cows excrete urines which carry only small amounts of the latter element; about 2 mg. per 100 cc. The urine of calves normally contains from fifteen to twenty times this amount.

The Effect of Individual Foods upon Blood and Urine of Cows.

It was my privilege while working under the direction of Dr. Lafayette B. Mendel to demonstrate the specific effect of individual foods upon the composition of the urine of men (1914). This study revealed the ease with which urinary acidity could be varied by changes in diet. Sherman and Gettler (1912) had previously made more reliable analyses of the ash of most of the common articles of food than had hitherto been available. Their analyses showed a preponderance of basic elements in the ash of vegetables and fruits, and an excess of acid-forming elements in that of meats and cereals. The result of eating the latter is an increased output of acid in the urine, but fruits and vegetables cause the production of less acid or alkaline urines. It was found that certain fruits, such as cranberries, plums, and prunes, although they yield an alkaline ash, nevertheless in metabolism led to an increase in urinary acidity. This anomalous result is doubtless, in part, due to the benzoic acid radical contained in these fruits.

Whether the alkaline reserve of the body can be altered by varying the intake of foods is an open question. Van Slyke and Cullen (1917) state that unusually high values for plasma CO_2 capacity are sometimes observed after the subject has partaken of an alkaline diet. Schloss and Harrington (1919) were able to increase the plasma CO_2-combining power of infants by appropriate changes in diet. On the other hand, McClendon, von Meysenbug, Engstrand, and King (1919) were unable to produce any change in the alkaline reserve of man by varying the diet.

In Tables III and IV are presented the analytical results of experiments which were designed to throw light on this question as well as on several others.

The first experiment on Cow 56 reveals very clearly the changes in the composition of blood and urine that may be produced by alterations in diet. It may, incidentally, be remarked that this cow was previously fasted for a period of 7 days. Her recovery from the fast was rapid and she was in excellent physical condition at the beginning of and during the present studies. This animal was first placed upon a diet of grain only.

Reference to Table III shows that on March 1, the 4th day on this food, her plasma CO_2 capacity was 58.6 cc. This value falls near the lower limit of those obtained for cows on mixed rations.

TABLE III.

Changes in Blood and Urine Produced by Individual Foods. Cow 56.

Date.	Plasma.					Urine.				Remarks.
	CO_2 capacity per 100 cc.	Ca per 100 cc.	Total P per 100 cc.	Inorganic P per 100 cc.	Lipoid P per 100 cc.	Specific gravity.	CO_2 per 100 cc.	NH_3 N per 100 cc.	P per 100 cc.	
1920	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*		*cc.*	*mg.*	*mg.*	
Mar. 1	58.6	10.3	12.2	5.7	7.5	1.027	37	11	178	Grain only.*
" 4	69.2	11.3	9.6	3.9	5.7	1.030	554	4	3	Alfalfa hay only.†
" 9	54.8	10.4	9.1	3.6	5.5	1.021	4	13	54	Corn silage only.‡

* Amounts of grain eaten:

	lbs.	
Feb. 27	10	(No. 17)
" 28	18	(No. 17)
" 29	15½	(No. 17)
Mar. 1	12	(No. 18)

Composition of mixture No. 17:

Ground oats	100 parts
Bran	100 "
Hominy	100 "
Linseed meal	50 "

† Amounts of alfalfa hay eaten:

	lbs.
Mar. 1	5
" 2	20
" 3 and 4	20½

Composition of mixture No. 18:

Ground oats	100 parts
Corn-meal	100 "
Bran	100 "
Linseed meal	50 "
Cotton-seed meal	50 "

‡ Amounts of corn silage eaten:

	lbs.
Mar. 5	20
" 6	16
" 7	30
" 8	46
" 9	26

Immediately following the acid-forming grain diet, the cow was given alfalfa hay only. Blood taken on March 4 shows that the CO_2 chemically bound by 100 cc. of plasma had increased to a value of 69.2 cc., a change of 10.6 cc. Another sample, obtained on March 9, shows that when corn silage had been the sole food plasma CO_2 capacity decreased to a value of 54.8 cc., lower than that produced by the eating of grain. This last result seems, at first sight, rather surprising but upon study a satisfactory explanation can, I think, be given. Discussion of this point will be deferred to a later part of this communication.

The response of the plasma phosphorus compounds is significant. As a result of the high phosphorus, grain diet, high values for inorganic and lipoid phosphorus were produced. This is a corroboration of the phenomenon previously described by Meigs, Blatherwick, and Cary (1919, *a*). With a change to the low phosphorus food, hay, the inorganic phosphorus is seen to have dropped from 5.7 to 3.9 mg. in 100 cc. of plasma. Still further reductions in plasma phosphorus compounds resulted when corn silage was the sole food. Comparable changes in plasma phosphate may be observed in the case of Cow 66, Table IV.

The response of plasma calcium to changes in food is also noteworthy. When alfalfa hay with its high content of this element was the food, calcium amounted to 11.3 mg. per 100 cc. of plasma, as compared with 10.3 and 10.4 mg. on grain and silage. I have observed another case in which the concentration of calcium was high in an animal fed solely upon hay. In this instance an effort was being made to dry up hastily a cow which was nearly at the end of the gestation period. On the day prior to and on the day of taking the blood sample, this cow ate neither grain nor silage but consumed 16 pounds of alfalfa hay daily. Her plasma contained 12.1 mg. of calcium in 100 cc., as contrasted with the normal value of about 10 mg. These results are in harmony with those of Boggs (1908) who observed an increase of 36 per cent in the calcium content of dog blood as a result of feeding calcium lactate. It would appear that the level of plasma calcium may be influenced by the calcium content of the diet, but in cows on rations quite similar in this respect the concentration is subject to only small variations.

The changes produced by diet in certain constituents of the urine are most interesting. Urine resulting from the grain diet showed an excretion of 37 cc. of CO_2 in 100 cc. With a change to the base-forming food, hay, fifteen times this amount was found (554 cc.). Coincident with an acid urine excreted when the animal ate silage, only 4 cc. of CO_2 were found, less than in

TABLE IV.

Changes in Blood and Urine Produced by Individual Foods. Cow 66.

Date.	Plasma.				Urine.					Remarks.
	CO_2 capacity per 100 cc.	Total P per 100 cc.	Inorganic P per 100 cc.	Lipoid P per 100 cc.	Specific gravity.	CO_2 per 100 cc.	NH_3 N per 100 cc.	Ca per 100 cc.	P per 100 cc.	
1919	*cc.*	*mg.*	*mg.*	*mg.*		*cc.*	*mg.*	*mg.*	*mg.*	
Oct. 16	60.7	10.3	4.9	5.4	1.035	32	25		2.7	Silage 16 lbs., grain 2 lbs.
" 20	64.1	10.0	5.0	5.0		306	6		1.7	Alfalfa hay only.*
" 31	64.5	9.9	4.6	5.3	1.037	358	6	4.8	2.4	Silage 16 lbs., grain 2 lbs.
Nov. 4	63.6	11.1	6.1	5.0	1.035	142	12	3.2	20.2	Grain only.†
" 17	67.3	8.7	3.9	4.8	1.037	385	5	12.0	2.2	Silage 16 lbs., grain 2 lbs.
" 20	61.7	8.2	2.9	5.3	1.036	200	9	50.5	2.5	Silage only.‡

* Amounts of alfalfa hay eaten:

	lbs.
Oct. 16	12
" 17	8¾
" 18	11½
" 19	8
" 20	6¼

† Amounts of grain eaten: Composition of grain mixture:

	lbs.		
Nov. 1	10	Ground oats	100 parts
" 2	14	Hominy	100 "
" 3	18	Bran	100 "
" 4	6	Linseed meal	50 "

‡ Amounts of silage eaten:

	lbs.
Nov. 17	12
" 18	30
" 19	58
" 20	4½

the case of grain. Appropriate and corresponding values for ammonia were observed; *i.e.*, 11, 4, and 13 mg. of N in 100 cc. of urine. The urinary excretion of phosphorus reveals some noteworthy features. Upon the high phosphorus, acid-forming, grain diet this cow excreted 178 mg. of P in 100 cc. of urine. This value became 3 mg. in the urine formed from the low phosphorus, base-forming hay. Although the urine from the silage period was more acid than that from the grain, still less than one-third as much phosphorus (54 mg.) was eliminated in this excretion. These figures show conclusively that phosphorus contained in the grain was much more efficiently and rapidly absorbed than it was from either of the other foods.

These and other experiments demonstrate that the amount of phosphorus excreted in the urine is dependent upon the quantity of phosphate in the plasma. But this relation is disturbed when an increased acid production occurs. This is well illustrated by comparing the values obtained from the hay and silage periods. Although the inorganic phosphorus of the plasma was lower when silage was fed, nevertheless, eighteen times as much phosphorus was excreted in this urine owing to the necessity for the elimination of acid. Another factor which doubtless contributed to the low value of 3 mg. in the urine of the hay period was the presence of large amounts of calcium in the food.

The other experiment on Cow 66 was arranged differently. She was placed upon a basal ration of 16 pounds of silage and 2 pounds of grain daily, and after she ate this ration for 4 to 5 days blood and urine samples were obtained. The animal then received only one food, for instance hay, during a period of 3 to 4 days when samples of blood and urine were again taken. Between the experimental periods, the cow was given the basal ration of 16 pounds of silage and 2 pounds of grain. It developed that this basal ration was insufficient for maintenance and the experimental findings must be interpreted with this reservation in mind.

If we turn our attention to the results shown in Table IV, we will observe that the values obtained for the three sets of samples resulting from the basal ration are not comparable. The first samples obtained on October 16 show evidences of acidosis; *i.e.*, low urinary CO_2 and a considerable amount of ammonia. The second and last specimens of urine corresponding to the basal ration are quite similar to each other as is seen by comparing the values for urinary CO_2 and ammonia. These latter values are evidently the correct ones for this basal diet. For some unknown reason, the animal was mildly acidotic when the first samples were obtained.

In spite of the fact that this cow ate but sparingly of alfalfa hay, $11\frac{1}{2}$ pounds in 1 day being the maximum, the CO_2 capacity changed from 60.7 to 64.1 cc. per 100 cc. of plasma, the neutral urine became strongly alkaline, urinary CO_2 increased from 32 to 306 cc., and ammonia N decreased from 25 to 6 mg. in 100 cc. of urine. The large excess of base in alfalfa hay most efficiently overcame the slight acidosis indicated by the first sample.

The effect of eating grain only is seen in the second experimental period. There is a slight decrease in plasma CO_2 capacity. The urine has become less alkaline, CO_2 has dropped from 358 to 142 cc., ammonia has doubled in amount, and phosphorus has increased from 2.4 to 20.2 mg. in 100 cc. The effect of the excess of acid-forming elements in grain is here seen chiefly in the change in urinary composition.

In the last period, when corn silage was the sole food, evidences of increased acid production are seen at several points. The CO_2-combining power of plasma showed a decrease from 67.3 to 61.7 cc., the urinary CO_2 dropped from 385 to 200 cc., and the ammonia nearly doubled in amount. The behavior of the phosphorus fraction of the blood plasma in this set of experiments helps to clarify some of the apparent mysteries. It will be observed that the total phosphorus decreased nearly 16 per cent from October 16 to November 20. This change took place in both the inorganic and the lipoid fractions. Since these changes were found when the animal was on the same ration, some fundamental cause must be sought in explanation. The level of inorganic phosphorus in blood plasma has come to be regarded in this laboratory as a fairly reliable indication of an animal's nutritive condition. It was previously stated that the basal ration of silage and grain was inadequate for maintenance, and the decrease in phosphorus of the plasma is probably concrete evidence of this deficiency.

Some Effects of Fasting in the Cow.

The effects of fasting upon the composition of the blood and especially of the urine have been quite extensively investigated, particularly in the case of dogs and men. Such studies have contributed much of value to our knowledge of the metabolism of protein, fat, and carbohydrate, supplied solely from the subject's own body.

In order to gain a further understanding of the manner in which tissue neutrality in cattle is maintained, a fasting experiment of 7 days duration was carried through on a cow whose physical condition was excellent. This animal was neither pregnant nor lactating; her last calf was born 23 months prior to the beginning of the fast, and she had been dry for more than 13 months. These points are emphasized because of their probable bearing upon the course of the fast. It is evident that this cow had ample opportunity for building large reserves of tissue, and, what is doubtless of importance in this connection, of mineral constituents. During the experiment, the animal was kept in an ordinary stanchion and bedded with shavings. The fast may be considered complete, for she drank only 10 pounds of water throughout the fasting period of 7 days, although water was offered daily. The cow weighed 1,042 pounds at the beginning of the fast and 915 pounds when it closed, a loss representing more than 12 per cent of the original body weight.

Table V records the changes produced in blood plasma and urine by abstinence from food. Blood obtained about 5 hours after the last feeding of grain and silage on December 3 showed that 64.6 cc. of CO_2 were chemically bound by 100 cc. of plasma. This value, which is above the average of 61.5 cc. for cows living on mixed rations, shows that the animal was not acidotic and was in a good state for the subsequent fast. After 24 hours of fasting, the CO_2 capacity was 66.2 cc., indicating a slight increase in the alkaline reserve. The plasma CO_2 capacity remained at or near this latter figure throughout the period of inanition. Judging from the experience of Benedict (1915) in his investigation of the fasting man, Levanzin, who showed a decrease in the alveolar CO_2 tension on the 2nd fasting day and a further drop on the 14th day, one would have expected a lowering of the CO_2 capacity in this case. It is plain that the CO_2 capacity of the plasma gave absolutely no indication of an acidotic condition.

The only evidence of acidosis afforded by the plasma is obtained from the phosphorus fraction. At the end of the 1st fasting day the inorganic phosphorus had increased about 11 per cent in amount. This is probably to be interpreted as an early mobilization of the mineral reserves to assist in the maintenance of tissue neutrality. Inorganic phosphorus of the plasma remained at this higher level until the last day when it fell to a value lower than that obtained on the feeding day. In other short time fasting experiments of about 38 hours duration, conducted in this laboratory, larger increases in the phosphate of the plasma have been found; *i.e.*, 11, 24, and 81 per cent.[1]

TABLE V.

Plasma and Urine of a Fasting Cow.

Date.	Day of fast.	Plasma.					Urine.				
		CO_2 capacity per 100 cc.	Ca per 100 cc.	Total P per 100 cc.	Inorganic P per 100 cc.	Lipoid P per 100 cc.	Specific gravity.	CO_2 per 100 cc.	NH_3 N per 100 cc.	Ca per 100 cc	P per 100 cc.
1919		*cc.*	*mg.*	*mg.*	*mg.*	*mg.*		*cc.*	*mg.*	*mg.*	*mg.*
Dec. 3	*	64.6	10.4	9.2	4.7	4.5	1.038	394	6	4.6	16.1
“ 4	1	66.2	10.0	10.5	5.2	5.3	1.032	359	7	3.2	20.6
“ 5	2	67.2					1.033	79	10		
“ 6	3	66.9	10.2	10.3	5.3	5.0	1.029	68	10	2.4	177.2
“ 7	4	66.5					1.029	29	13		
“ 8	5	65.5	10.0	9.7	5.2	4.5				2.2	217.2
“ 9	6	66.4									
“ 10	7	66.0	10.4	8.2	4.2	4.0	1.027			1.9	156.3

* The rations of this cow prior to the fast were:

		lbs.		*lbs.*		*lbs.*
From Nov. 23 to 29...........	Bran	6	Soy bean hay	2	Silage	24
Nov. 30.......................	“	6	“ “ “	2	“	21
Dec. 1 to 2	“	6	“ “ “	2	“	18
“ 3, a.m..................	“	3			“	11

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[1] I am indebted to Dr. E. B. Meigs and Dr. C. A. Cary for permission to quote these unpublished results.

A change in the lipoid phosphorus of the plasma also occurred. After one day of inanition, there was an increase of about 18 per cent in this constituent. A gradual decrease in phosphatide phosphorus is seen throughout the remainder of the fast. The early augmentation of this fraction of the phosphorus content of the plasma is probably an expression of the quick mobilization of body fat, a phenomenon quite comparable to the increase observed in lactation, particularly in its earlier stages when there is a tendency for body fat to be mobilized and thrown into the blood stream, as suggested by Meigs, Blatherwick, and Cary (1919, *a*).

The calcium of the plasma showed small variations which lie within the limits of experimental error.

Turning our attention to the urine, the characteristic changes produced by lack of food are seen. The first urine obtained after the cow had eaten her last food gave the typical strongly alkaline reaction, with a large amount of CO_2 (394 cc)., small ammonia N (6 mg.), and a phosphorus content of 16.1 mg. in 100 cc. There was a slight change in the direction of decreasing alkalinity already evident at the end of the first fasting day, but this did not become well marked until the 2nd day, and on the 3rd day the CO_2 was 88 per cent less, ammonia 66 per cent more, and phosphorus ten times greater than in the original urine. The urine of the 4th day revealed a still further drop in CO_2 and an increase in ammonia. This latter sample was the first obtained by catheter, and the procedure unfortunately started a bladder condition which rendered determinations of CO_2 and ammonia of subsequent urines valueless. However, one sees that the elimination of acid had not yet reached its highest point, for the urine of the 5th day contained 217 mg. of phosphorus in 100 cc., as compared with 16 mg. in that of the control day. It would seem that the urinary elimination of phosphorus had reached its height about the 5th day, as the only remaining determination shows that this element had decreased to a value of 156 mg. in 100 cc. of the urine of the last day.

Experimental Acidosis in a Calf.

In Table VI are given the analytical data obtained from the plasma and urine of a calf which was rendered acidotic by feeding hydrochloric acid. This was one of the experiments planned to throw light on the hypothesis that plasma calcium is dependent upon the bicarbonate content of this fluid. At 2 days of age the animal showed a CO_2 capacity of 68.3 cc. and a calcium content of 12.1 mg. per 100 cc. of plasma. About 1 month later these values had become 61.4 cc. and 11.1 mg., respectively. After this sample of blood was taken on September 27, 10 gm. of $NaHCO_3$

TABLE VI.

Experimental Acidosis in a Calf.

Date.	Plasma.					Urine.			Remarks.
	CO_2 capacity per 100 cc.	Ca per 100 cc.	Total P per 100 cc.	Inorganic P per 100 cc.	Lipoid P per 100 cc.	Specific gravity.	CO_2 per 100 cc.	NH_3 N per 100 cc.	
1919	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*		*cc.*	*mg.*	
Aug. 29	68.3	12.1							2 days old.
Sept. 27	61.4	11.1	12.7	7.4	5.3	1.006	5.7	17	Up to date 12 lbs. of whole milk daily.
" 30						1.011	63.5	9	Sept. 28, 29, 30; 10 gm. of $NaHCO_3$ daily.
Oct. 2	66.4	11.2	13.8	8.2	5.6	1.005	51.7	4	Oct. 1; 20 gm. of $NaHCO_3$. Oct. 2, a.m.; 15 gm.
" 6						1.009	0.6	36	Oct. 3 and after; 180 cc. of 1 N HCl daily.
" 8	48.5	11.7	12.2	8.0	4.2	1.010	0.2	67	Oct. 6; 180 cc. of 1 N HCl. Oct. 7; 240 cc. Oct. 8, a.m.; 180 cc. P in urine of Oct. 8, 75 mg. per 100 cc.

were added to the feed of 12 pounds of whole milk daily. On October 1, 20 gm. of $NaHCO_3$ were given and on the following morning it received 15 gm. of this substance. Blood taken on this date revealed the characteristic increase in CO_2 capacity. The urine was alkaline, its CO_2 had increased from 6 to 52 cc., while the ammonia decreased from 17 to 4 mg. of N per 100 cc. The calf was immediately changed to an acid diet, receiving with milk 180 cc. of 1 N HCl daily from October 3 to 6 inclusive. On the following day the dose was increased to 240 cc., and on the morning of the 8th it drank 180 cc. of this acid. A sample of blood obtained on this latter date showed that the plasma CO_2 capacity had responded with the well known drop to 48.5 cc. The corresponding urine was decidedly acid, its CO_2 had practically disappeared, and the ammonia had increased to 67 mg. of N in 100 cc.

Regarding the hypothetical relation between plasma calcium and bicarbonate this experiment offers slight positive evidence. A decrease in CO_2 capacity from 61.4 to 48.5 cc. was accompanied by an increase in calcium from 11.1 to 11.7 mg. per 100 cc. of plasma. However, another experiment on a calf 2 weeks of age resulted in a decrease of calcium from 11.5 to 11.0 mg. when the CO_2 capacity was lowered from 73.0 to 54.1 cc. by feeding hydrochloric acid. These findings indicate that the level of plasma calcium is very little or not at all influenced by physiological changes in the alkaline reserve.

Some Effects of Feeding Benzoic Acid to a Calf.

One of the striking differences in the metabolism of calves and cows is found in the small excretion of hippuric acid in the urine of young animals. It is only when the latter begin to eat foods other than milk that appreciable amounts of this acid appear. Among the foods consumed by herbivora which give rise to the formation of hippuric acid, meadow hay was observed by Weiske (1876) to be the most efficient. This author believed that most of the hippuric acid precursor was found in the weeds which contaminated the hay. He also observed that wheat and oat straws lead to the formation of considerable amounts of hippuric acid, but none of this acid was excreted when the experimental animals, wethers, were fed pea straw, bean straw, unpeeled potatoes, peas, linseed, wheat, or oats.

It seemed to be an interesting point to discover how a young calf would react to benzoic acid when fed along with a non-hippuric acid-forming diet. A calf, aged 1 month, was accordingly placed upon a uniform milk diet to which a certain amount of benzoic acid was added. On February 16, samples of blood and urine resulting from the milk diet were obtained. Normal values for a calf of that age are shown in Table VII. Plasma CO_2 capacity, 71.0 cc., was somewhat above the higher adult figures. The urine was neutral in reaction and contained 9.7 cc. of CO_2, 14.6 mg. of ammonia N, and 47.3 mg. of P in 100 cc. Urine obtained the following day after the calf had received 10 gm. of benzoic acid was acid to litmus, and contained only 1.4 cc. of CO_2 in 100 cc., and the ammonia had nearly tripled in value. After having received a total of 20 gm. of the acid, the plasma CO_2 capacity of the 2nd day decreased slightly to 68.0 cc. per 100 cc. The corresponding urine was decidedly acid, CO_2 was the same as on the preceding day, ammonia N increased to 71.6 mg., and phos-

TABLE VII.

Composition of Blood and Urine of Calf Fed Benzoic Acid.

Date.	Plasma.					Urine.				Remarks.
	CO_2 capacity per 100 cc.	Ca per 100 cc.	Total P per 100 cc.	Inorganic P per 100 cc.	Lipoid P per 100 cc.	Specific gravity.	CO_2 per 100 cc.	NH_3 N per 100 cc.	P per 100 cc.	
1920	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*		*cc.*	*mg.*	*mg.*	
Feb. 16	71.0	10.5	10.3	7.5	2.8	1.007	9.7	14.6	47.3	Whole milk diet. 5 gm. of benzoic, p.m.
" 17						1.012	1.1	39.3		10 gm. of benzoic.
" 18	68.1	9.9	10.4	7.6	2.8	1.015	1.4	71.6	142.1	5 gm. of benzoic, a.m., 5 gm. of benzoic, p.m.
" 19						1.008	2.5	30.5		5 gm. of benzoic, a.m., 10 gm. of benzoic, p.m.
" 20	71.1	10.4	10.4	7.7	2.7	1.008	1.6	26.7	58.7	10 gm. of benzoic, a.m.

phorus was three times larger than in the first sample. The results thus far indicated the possibility of producing an acidosis somewhat comparable to that resulting from the use of mineral acids. It was, therefore, decided to push the dosage of the acid. Urine obtained on the following morning gave values for CO_2 and ammonia which pointed to a return to normal conditions. On the afternoon of February 19, the dose was increased to 10 gm. In spite of this larger amount of acid, samples of blood and urine secured the next morning revealed values for plasma CO_2 capacity, urinary ammonia, and phosphorus which were nearly comparable with those obtained before the administration of benzoic acid.

It is evident that a condition of acidosis was produced when benzoic acid was first administered, but that nearly complete recovery followed in spite of larger doses of the acid. During the period of acidosis, the calf was suffering from diarrhea, but the feces were normal in appearance on the last day of the experiment. How this digestive disturbance may have influenced the results is not known. Determination of the hippuric and benzoic acid content of the blood and urine would, no doubt, have aided in the interpretation of the experimental results.

DISCUSSION.

The experiments dealing with changes in blood and urine resulting from variations in diet leave no question, it seems to me, that the ability of plasma to bind carbon dioxide chemically may be altered by the ingestion of foods of different potential acidity or alkalinity. This is very clearly shown in the case of Cow 56, Table III. The CO_2 capacity of her plasma, after being fed solely on an acid-forming grain mixture was 58.6 cc. in contrast with a value of 69.2 cc. when she ate the strongly base-forming alfalfa hay. Similar responses of the plasma alkaline reserve to changes in diet, although less marked, are evident in the case of Cow 66, Table IV.

The ability of a diet of corn silage, the ash of which contains an excess of basic elements, to decrease plasma CO_2 capacity and to cause the formation of acid urines is a most interesting phenomenon. This effect is definitely shown in the case of Cow 56, Table

III. After she had eaten heartily of this food, CO_2 chemically bound amounted to 54.8 cc. per 100 cc. of plasma. The corresponding urine was decidedly acid to litmus, and contained only 4 cc. of CO_2 in 100 cc., together with considerable quantities of ammonia and phosphorus. The reserve alkalinity of this animal was certainly reduced as the result of eating a food which is generally considered base-forming in character.

How may this unexpected result be explained? May not the organic acids of corn silage be responsible for this anomalous effect? According to Dox and Neidig (1912, 1913), 100 gm. of dried corn silage contain about 1.63 gm. of volatile acids of which acetic acid comprises 90 per cent. The chief remaining volatile acid is propionic. Butyric acid occurs in small amounts in good material and increases when spoilage occurs. The nonvolatile acid of silage is racemic lactic which is present in greater amounts than all other acids combined. The proportion of nonvolatile to volatile acids is approximately 100:75. If a calculation is made of the amount of organic acids in the silage consumed by Cow 56 in her last three meals before blood and urine samples were taken, it is found that she received about 373 gm. of organic acids distributed as follows: acetic 144 gm., other volatile acids 16 gm., and racemic lactic 213 gm. Of this total, slightly more than one-third was eaten the morning that samples were taken. Cow 66 during the corresponding period ate 62½ pounds of silage which would yield nearly as large an intake of organic acids (327 gm.). However, she ingested but 4½ pounds of silage on the morning that samples of blood and urine were obtained. The changes in her blood and urine though distinct were not nearly so marked as in the other case.

Further evidence bearing upon the point is the following: Cow 66 possessed a lower CO_2 capacity of the plasma; her urine contained less CO_2 and more ammonia when she ate silage alone than when smaller amounts of silage (16 pounds) with the addition of 2 pounds of grain, an acid-forming food, were consumed. A reasonable explanation of this phenomenon is made by assuming that a cow is able to oxidize certain amounts of the organic acids of silage, but when this limit is reached these acids act like a mineral acid in lowering the alkaline reserve and causing the elimination of acid urines. In the present state of our knowledge concerning the quantities of organic acids which the body is able to oxidize, one cannot hazard an opinion as to whether one or more of the acids found in silage are responsible for the effects observed. It is also probable that an increased formation of hippuric acid may be a factor contributing to this greater acid production.

Those authors who have been unable to change the alkaline reserve by altering the diet were probably unfortunate in their selection of experimental subjects, or the food consumption may not have been sufficiently great to produce the effect.

Further study of the calcium content of the blood plasma of cattle confirmed the observations previously reported from this laboratory. The plasma of young calves contains more of this element than does that of cows. In adult animals the level of plasma calcium tends to remain quite uniform but it may be altered somewhat by appropriate changes in the amount supplied with the rations. Experiments designed to throw light on the hypothesis that the amount of calcium in plasma is dependent upon the bicarbonate content of this fluid failed to demonstrate any such relation when the changes in bicarbonate were within physiological limits. These results are not entirely in harmony with those of Allers and Bondi (1907) who state that they were able to double the calcium content of the blood of rabbits by feeding hydrochloric acid. Their doses were proportionally much larger than mine, and it seems probable that feeding acid has no effect on plasma calcium unless sufficiently large amounts are given to produce a great depletion in the alkaline reserve.

In cattle the regulation of tissue neutrality assumes a different character than in omnivora or carnivora. The primary concern of the cow is to rid the body of excess base which is normally so abundantly furnished by the food. The problem, therefore, is generally quite the opposite of that which relates to non-herbivorous animals. Accordingly one finds the cow excreting a strongly alkaline urine which contains large amounts of carbonate, equivalent in many instances to about 40 gm. of CO_2 daily. Only small quantities of ammonia and phosphorus are found owing to small need for the elimination of acid. When the natural inclinations for food are interfered with by restricting the animal to an acid-forming food, by fasting, or by feeding acid *per se*, an approach to the conditions obtaining in man or carnivora is manifest. Under these circumstances the body clings to its alkali, as indicated by a decreased CO_2 output in the urine. Likewise, increased amounts of urinary ammonia and phosphorus appear in response to the necssity for increased acid excretion.

Calves, on the other hand, approach more nearly the conditions which obtain in man. While calves live on an approximately neutral diet of milk, their urines are normally neutral in reaction, and contain small amounts of carbonate, and significantly greater quantities of ammonia and phosphorus. In spite of these differences in the urines of young and adults, the carbon dioxide-combining power of the plasma of calves is decidedly higher than that of cows. It is clear that a fundamentally different regulatory mechanism is functioning in the two cases. A comparison of the hydrogen ion concentration of the two bloods, and a study of the respiration of young and adults should prove valuable aids to a further elucidation of this problem.

Study of the carbon dioxide capacity of cow plasma shows that a determination of this value is of little aid in detecting mildly acidotic conditions. Although the variations in the ammonia content of urine are significant, many complicating factors enter to make an estimation of this constituent not entirely suitable for an indicator of increased acid production. Apparently, the most reliable indication of acidosis in cattle is found in the output of carbonate in the urine. Decreased amounts of urinary CO_2 are the first expression by the cow of an increased acid metabolism. This reaction is quickly evident, whereas rather forced conditions must prevail before large changes in plasma CO_2 capacity are produced.

SUMMARY.

The alkaline reserve of the cow, as measured by the carbon dioxide-combining power of the plasma, is remarkably constant. Twenty-two determinations of the plasma CO_2 capacity of sixteen individuals gave an average value of 61.5 cc. per 100 cc. with maximum and minimum figures of 68.3 and 55.1 cc., respectively.

The amount of CO_2 chemically bound by the plasma of young calves is decidedly greater than by that of adult animals. The average CO_2 capacity of the plasma of seven calves ranging in

age from 2 to 14 days was 73.0 cc. Maximum and minimum values for the same calves were 80.6 and 68.3 cc. per 100 cc.

Significant changes in the alkaline reserve of cows were produced by altering the diet with respect to the acidity or alkalinity of its ash. Corn silage, a food generally considered to be base-forming in character, when fed in large amounts to a cow decreased the plasma CO_2 capacity and led to the formation of an acid urine. Reasons were advanced for believing that this anomalous result was due to the incomplete oxidation of the organic acids of silage.

A cow that was fasted for 7 days failed to show any decrease in plasma CO_2 capacity. An increase in the inorganic phosphorus of the plasma was interpreted as an expression of the mobilization of the mineral reserves to aid in maintaining tissue neutrality.

The amount of calcium in the plasma of cattle may be influenced by the quantities in the ration but the changes are not great even when large variations are made in the intake. Under physiological conditions, the level of plasma calcium appears to be very little, if at all, dependent upon the bicarbonate content of this fluid.

Comment was made upon some of the differences between herbivora and carnivora or omnivora with respect to the maintenance of tissue neutrality. In this connection, the practicability of measuring the CO_2 content of the urine of herbivora to determine changes in the excretion of base was emphasized.

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EFFECT OF ANESTHETICS ON VARIOUS CELL ACTIVITIES.

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Experiments on the Aquatic Plant, Elodea.

In order to determine CO_2 content of the medium from the pH, the solution used in the experiments was 0.0025 N $NaHCO_3$, brought first to pH 7.6 by adjusting the CO_2 content. A change in pH of 0.1 indicated a change in CO_2 content of 1.5 cc. per liter. The pH was determined by comparison with a set of standards prepared from Sörensen's phosphate mixtures, colored with phenolsulfonephthalein, and sealed in Nonsol test-tubes 25 mm. in diameter.

For oxygen determinations, a terminal sprig of *Elodea*, 8 inches long, was washed in the bicarbonate solution, placed in a 100 cc. bottle filled with the same solution, and the bottle enclosed in an opaque container sunk in the thermostat. The feathery nature of this plant facilitates exchange with the medium. At the same time another 100 cc. bottle was filled with the solution without the *Elodea*. At the end of 3 hours the plant was pulled out with forceps and the solutions in the two bottles were titrated by the Winkler method. The difference in the two titrations gave the O_2 consumption. The same sprig of *Elodea* was employed in a similar manner for determination of O_2 consumption in the bicarbonate solution to which the anesthetic had been added. A second sprig of the plant was used to determine change in pH and exosmosis, the contents of the 100 cc. bottle being used to fill the tubes for the two determinations. The exosmosis of chlorides was determined with a Richard nephelometer. Photosynthesis was determined similarly to respiration except that the opaque container was omitted and a 240 watt stereopticon lamp was placed 1 foot from the plant. The heat of the lamp was screened off by a layer of water. Protoplasmic rotation was determined by means of a micrometer eyepiece in a microscope.

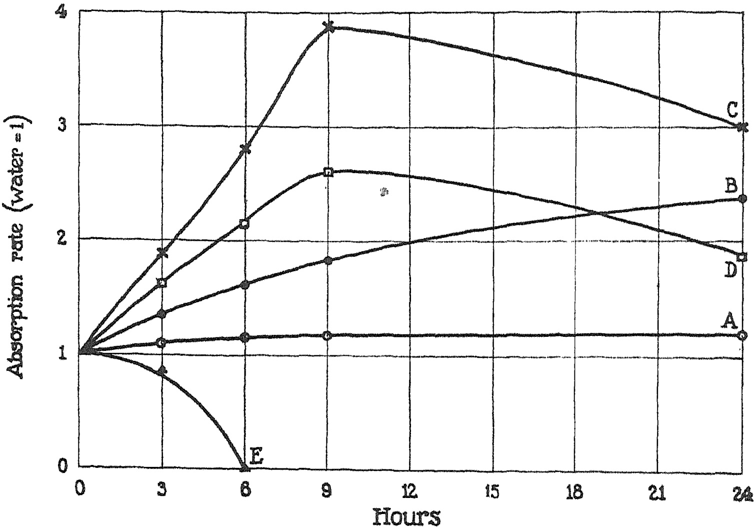

FIG. 1. Relative rate of O_2 absorption of *Elodea* in solutions of alcohol. Curve A, 1 per cent; B, 1.5 per cent; C, 3 per cent; D, 6 per cent; E, 15 per cent (Table I).

The experiments were made in a thermostat at 30°, the best record of which showed a fluctuation of 0.01° in 1 day and a continuous deviation amounting to 0.1° in 10 days. Since a variation of 1° means an error of 25 per cent, it was necessary that respiration with and without the anesthetic be carried on at the same temperature.

A 1 per cent solution of alcohol (Fig. 1, Curve A) produced practically no effect on rate of O_2 consumption. Even after 3 days the rate showed no decrease (1.21). In the 1.5 per cent solution (Curve B) the rate showed a steady increase for 24 hours. When such a piece was kept in the alcohol 2 days longer, the rate remained practically the same as at the close of 24 hours. If it was returned to water, the rate seemed to increase still more rapidly, and to continue so for at least 3 days. In one instance the rate after 3 days in tap water following 24 hours in 1.5 per cent alcohol was 2.86. This is in harmony with the fact that slight anesthetization hastens the opening of dormant buds on deciduous plants.

In the 3 per cent solution (Curve C) the rate increased still more rapidly, reaching 3.87 at the close of 9 hours, but by the end of 24 hours had decreased to 2.99 (Table I, No. 4). In 6 per cent solution (Curve D) the increase was not so great, and the point reached at the close of 24 hours was correspondingly lower. In the 15 per cent solution (Curve E) there was no increase. Since there existed the possibility that an increase may have occurred, lasting for a much briefer period than that employed in this instance, the experiment was repeated and determinations were made after intervals of 20 minutes (Table II).

No increase of O_2 consumption was discernible even when the periods were reduced to 20 minutes. The experiment was repeated using 10 minute intervals, but, although the differences were so slight as to be inconclusive, they indicated that no decrease of rate was occurring.

The curves obtained from measurement of changes of pH due to CO_2 were very similar to those of O_2 consumption in solutions of low concentration (alcohol 1, 1.5, and 3 per cent, Table III and Fig. 2). But 6 and 15 per cent alcohol caused acceleration of rate at first, the latter producing the maximum change.

In all the solutions of alcohol, except the 1.0 per cent solution, the rate of exosmosis of chlorides from the cell increased (Table IV and Fig. 3). This acceleration was very slight, however, in all cases except the 6 and 15 per cent solutions. It was especially marked in the latter where the cells were so injured that after 15 minutes immersion irreversible plasmolysis occurred.

In the 15 per cent solution protoplasmic rotation had ceased entirely (Table V and Fig. 4) within 1 minute, and after 15 minutes was irreversible. In the 6 per cent solution the decrease in rate was gradual; in the 3 and 1.5 per cent solutions an increase was shown. If these tips of *Elodea* were returned to water after

TABLE I.

Respiration as Measured by the Amount of Oxygen Extracted by Elodea from Solutions.

9 hrs. in water and 24 hrs. in solution. Readings taken after 3 hr. periods in water, and in anesthetics at the close of 3, 6, and 9 hrs. immersion, and after the period extending from 21 to 24 hrs. At the beginning of each 3 hr. period, the plant was placed in a fresh solution. For corresponding curves see Figs. 1, 5, 10, and 14.

Plant No.	Solution.	Concentration.	Time.	Thiosulfate.	Change.	Relative rate of O_2 absorption.
		per cent	*hrs.*	*cc.*	*cc.*	
1	Water.		1–3	2.25–1.83	0.42	
	“		3–6	2.25–1.84	0.41	
	“		6–9	2.25–1.83	0.42	
	“		21–24	2.25–1.83	0.42	
2	“		1–3	2.25–1.85	0.40	
	“		3–6	2.25–1.85	0.40	
	“		6–9	2.25–1.85	0.40	
	Alcohol.	1	1–3	2.25–1.80	0.45	1.12
	“	1	3–6	2.25–1.78	0.47	1.16
	“	1	6–9	2.25–1.78	0.47	1.16
	“	1	21–24	2.25–1.77	0.48	1.20
3	Water.		1–3	2.25–1.85	0.40	
	“		3–6	2.25–1.85	0.40	
	“		6–9	2.25–1.85	0.40	
	Alcohol.	1.5	1–3	2.25–1.71	0.54	1.350
	“	1.5	3–6	2.25–1.61	0.64	1.600
	“	1.5	6–9	2.25–1.52	0.73	1.825
	“	1.5	21–24	2.25–1.30	0.95	2.375
4	Water.		1–3	2.25–1.80	0.45	
	“		3–6	2.25–1.80	0.45	
	“		6–9	2.25–1.79	0.46	
	Alcohol.	3	1–3	2.25–1.40	0.85	1.89
	“	3	3–6	2.25–0.99	1.26	2.80
	“	3	6–9	2.25–0.51	1.74	3.87
	“	3	21–24	2.25–0.91	1.34	2.99
5	Water.		1–3	2.25–1.85	0.40	
	“		3–6	2.25–1.85	0.40	
	“		6–9	2.25–1.85	0.40	
	Alcohol.	6	1–3	2.25–1.60	0.65	1.63
	“	6	3–6	2.25–1.39	0.86	2.15
	“	6	6–9	2.25–1.20	1.05	2.63
	“	6	21–24	2.25–1.50	0.75	1.88

TABLE I—*Continued.*

Plant No.	Solution.	Concentration.	Time.	Thiosulfate.	Change.	Relative rate of O_2 absorption.
		per cent	*hrs.*	*cc.*	*cc.*	
6	Water.		1–3	2.25–1.83	0.42	
	“		3–6	2.25–1.83	0.42	
	“		6–9	2.25–1.83	0.42	
	Alcohol.	15	1–3	2.25–1.90	0.35	0.83
	“	15	3–6	2.25–2.25	0.00	0.00
7	Water.		1–3	2.25–1.75	0.50	
	“		3–6	2.25–1.77	0.48	
	“		6–9	2.25–1.75	0.50	
	Ether.	1.5	1–3	2.25–1.50	0.75	1.50
	“	1.5	3–6	2.25–0.80	1.45	2.90
	“	1.5	6–9	2.25–1.45	0.90	1.80
	“	1.5	21–24	2.25–1.60	0.65	1.30
8	Water.		1–3	2.25–1.52	0.73	
	“		3–6	2.25–1.50	0.75	
	“		6–9	2.25–1.50	0.75	
	Ether.	3	1–3	2.25–0.95	1.40	1.86
	“	3	3–6	2.25–1.15	1.10	1.46
	“	3	6–9	2.25–1.55	0.70	0.93
	“	3	21–24	2.25–2.25	0.00	0.00
9	Water.		1–3	2.25–1.60	0.65	
	“		3–6	2.25–1.60	0.65	
	“		6–9	2.25–1.60	0.65	
	Ether.	5	1–3	2.22–1.12	1.10	1.69
	“	5	3–6	2.22–1.44	0.78	1.20
	“	5	6–9	2.22–1.98	0.24	0.37
	“	5	21–24	2.22–2.22	0.00	0.00
10	Water.		1–3	2.25–1.55	0.70	
	“		3–6	2.25–1.55	0.70	
	“		6–9	2.25–1.55	0.70	
	Ether.	8	1–3	2.17–1.27	0.90	1.29
	“	8	3–6	2.17–2.17	0.00	0.00
11	Water.		1–3	2.25–1.90	0.35	
	“		3–6	2.25–1.91	0.34	
	“		6–9	2.25–1.91	0.34	
	Chloroform.	0.05	1–3	2.25–1.85	0.40	1.13
	“	0.05	3–6	2.25–1.70	0.55	1.57
	“	0.05	6–9	2.25–1.60	0.65	1.86
	“	0.05	21–24	2.25–1.40	0.85	2.43

TABLE I—*Concluded.*

Plant No.	Solution.	Concentration.	Time.	Thiosulfate.	Change.	Relative rate of O_2 absorption.
		per cent	*hrs.*	*cc.*	*cc.*	
12	Water.		1–3	2.25–1.80	0.45	
	"		3–6	2.25–1.80	0.45	
	"		6–9	2.25–1.79	0.46	
	Chloroform.	0.15	1–3	2.25–1.50	0.75	1.63
	"	0.15	3–6	2.25–1.70	0.55	1.20
	"	0.15	6–9	2.25–1.90	0.35	0.77
	"	0.15	21–24	2.25–2.05	0.20	0.44
13	Water.		1–3	2.25–1.85	0.40	
	"		3–6	2.25–1.84	0.41	
	"		6–9	2.25–1.85	0.40	
	Chloroform.	0.30	1–3	2.24–1.69	0.55	1.38
	"	0.30	3–6	2.24–2.24	0.00	0.00
14	Water.		1–3	2.25–1.95	0.30	
	"		3–6	2.25–1.95	0.30	
	"		6–9	2.25–1.95	0.30	
	Chloretone.	0.05	1–3	2.25–1.90	0.35	1.17
	"	0.05	3–6	2.25–1.70	0.55	1.83
	"	0.05	6–9	2.25–1.55	0.70	2.33
	"	0.05	21–24	2.25–1.40	0.85	2.83
15	Water.		1–3	2.25–1.85	0.40	
	"		3–6	2.25–1.84	0.41	
	"		6–9	2.25–1.85	0.40	
	Chloretone.	0.1	1–3	2.25–2.05	0.20	0.50
	"	0.1	3–6	2.25–2.15–	0.10+	0.25+
	"	0.1	6–9	2.25–2.15	0.10	0.25
	"	0.1	21–24	2.25–2.15+	0.10–	0.25–

TABLE II.

Oxygen Consumption by Elodea in 15 Per Cent Alcohol during Six 20 Min. Periods.

Solution.	Thiosulfate.	Change.	Relative rate of O_2 absorption.
	cc.	*cc.*	
Water.	2.15–1.90	0.25	
Alcohol.	2.15–1.93	0.22	0.88
"	2.15–2.03	0.12	0.48
"	2.15–2.10	0.05	0.20
"	2.15–2.15	0.00	0.00
"	2.15–2.14	0.01(?)	0.04(?)
"	2.15–2.15	0.00	0.00

TABLE III.

CO_2 Liberated as Measured by the Changes in pH of Solutions.

Plant immersed 9 hrs. in water and 24 hrs. in solution. Readings taken after 3 hr. periods in water, and in anesthetics at the close of 3, 6, and 9 hrs. immersion, and after the period extending from 21 to 24 hrs. At the beginning of each 3 hr. period, the plant was placed in a fresh solution. For corresponding curves, see Figs. 2, 6, 11, and 15.

Plant No.	Solution.	Concentration.	Time.	pH	Change.	Relative rate of CO_2 elimination.
		per cent	*hrs.*		*cc.*	
2	Water.		1–3	7.8 -7.6	0.20	
	"		3–6	7.8 -7.6	0.20	
	"		6–9	7.8 -7.6	0.20	
	Alcohol.	1	1–3	7.6 -7.4–	0.20+	1.00+
	"	1	3–6	7.6 -7.4–	0.20+	1.00+
	"	1	6–9	7.6 -7.4–	0.20+	1.00+
	"	1	21–24	7.6 -7.35	0.25	1.25
3	Water.		1–3	7.8 -7.6	0.20	
	"		3–6	7.8 -7.6	0.20	
	"		6–	7.8 -7.6+	0.20–	
	Alcohol.	1.5	1–3	7.6 -7.35	0.25	1.25
	"	1.5	3–6	7.6 -7.3	0.30	1.50
	"	1.5	6–9	7.6 -7.3	0.30	1.50
	"	1.5	21–24	7.6 -7.2	0.40	2.00
4	Water.		1–3	7.8 -7.55	0.25	
	"		3–6	7.8 -7.55	0.25	
	"		6–9	7.8 -7.55	0.25	
	Alcohol.	3	1–3	7.6 -7.25	0.35	1.4
	"	3	3–6	7.6 -7.20	0.40	1.6
	"	3	6–9	7.6 -7.20	0.40	1.6
	"	3	21–24	7.6 -7.25	0.35	1.4
5	Water.		1–3	7.8 -7.6	0.20	
	"		3–6	7.8 -7.6	0.20	
	"		6–9	7.8 -7.6	0.20	
	Alcohol.	6	1–3	7.6 -7.25	0.35	1.75
	"	6	3–6	7.6 -7.25	0.35	1.75
	"	6	6–9	7.6 -7.3	0.30	1.50
	"	6	21–24	7.6 -7.4	0.20	1.00
6	Water.		1–3	7.8 -7.6	0.20	
	"		3–6	7.8 -7.6	0.20	
	"		6–9	7.8 -7.6	0.20	
	Alcohol.	15	1–3	7.6 -7.2	0.40	2.0
	"	15	3–6	7.6 -7.4	0.20	1.0
	"	15	6–9	7.6 -7.6	0.00	0.0

TABLE III—*Continued.*

Plant No.	Solution.	Concentration.	Time.	pH	Change.	Relative rate of CO_2 elimination.
		per cent			*cc.*	
7	Water.		1–3	7.8–7.5	0.30	
	“		3–6	7.8–7.5	0.30	
	“		6–9	7.8–7.5	0.30	
	Ether.	1.5	1–3	7.8–7.45	0.35	1.17
	“	1.5	3–6	7.8–7.4	0.40	1.33
	“	1.5	6–9	7.8–7.4	0.40	1.33
	“	1.5	21–24	7.8–7.4	0.40	1.33
8	Water.		1–3	7.8–7.5	0.30	
	“		3–6	7.8–7.5	0.30	
	“		6–9	7.8–7.5	0.30	
	Ether.	3	1–3	7.8–7.4	0.40	1.33
	“	3	3–6	7.8–7.6	0.20	0.67
	“	3	6–9	7.8–7.7	0.10	0.33
	“	3	21–24	7.8–7.8	0.00	0.00
9	Water.		1–3	7.8–7.5	0.30	
	“		3–6	7.8–7.5	0.30	
	“		6–9	7.8–7.5	0.30	
	Ether.	5	1–3	7.8–7.25	0.40	1.83
	“	5	3–5	7.8–7.8	0.00	0.00
10	Water.		1–3	7.8–7.4	0.40	
	“		3–6	7.8–7.4	0.40	
	“		6–9	7.8–7.4	0.40	
	Ether.	8	1–3	7.7–6.8	0.90	2.25
	“	8	3–6	7.7–7.7	0.00	0.00
11	Water.		1–3	7.8–7.6	0.20	
	“		3–6	7.8–7.6	0.20	
	“		6–9	7.8–7.6	0.20	
	Chloroform.	0.05	1–3	7.5–7.3	0.20	1.0
	“	0.05	3–6	7.5–7.3	0.20	1.0
	“	0.05	6–9	7.5–7.25	0.25	1.25
	“	0.05	21–24	7.5–7.2	0.30	1.50
12	Water.		1–3	7.8–7.55	0.25	
	“		3–6	7.8–7.55	0.25	
	“		6–9	7.8–7.55	0.25	
	Chloroform.	0.15	1–3	7.7–7.2	0.50	2.0
	“	0.15	3–6	7.7–7.45	0.25	1.0
	“	0.15	6–9	7.7–7.45	0.25	1.0
	“	0.15	21–24	7.7–7.6+	0.10–	0.4–

TABLE III—*Concluded.*

Plant No.	Solution.	Concentration.	Time.	pH	Change.	Relative rate of CO_2 elimination.
		per cent	*hrs.*		*cc.*	
13	Water.		1–3	7.8 –7.6	0.20	
	"		3–6	7.8 –7.6	0.20	
	"		6–9	7.8 –7.6	0.20	
	Chloroform.	0.3	1–3	7.8– 7.2	0.60	3.0
	"	0.3	3–6	7.8 –7.4	0.40	2.0
	"	0.3	6–9	7.8 –7.6	0.20	1.0
	"	0.3	21–24	7.8 –7.8	0.00	
14	Water.		1–3	7.8 –7.6	0.20	
	"		3–6	7.8 –7.6	0.20	
	"		6–9	7.8 –7.6	0.20	
	Chloretone.	0.05	1–3	7.55–7.25	0.30	1.50
	"	0.05	3–6	7.55–7.15	0.40	2.00
	"	0.05	6–9	7.55–7.1	0.45	2.25
	"	0.05	21–24	7.55–6.95	0.60	3.00
15	Water.		1–3	7.8 –7.4	0.4	
	"		3–6	7.8 –7.4	0.4	
	"		6–9	7.8 –7.4	0.4	
	Chloretone.	0.1	1–3	7.65–7.00	0.65	1.62
	"	0.1	3–6	7.6 –6.85	0.8	2.00
	"	0.1	6–9	7.6 –6.95	0.7	1.75
	"	0.1	21–24	7.6 – .35	0.3	0.75

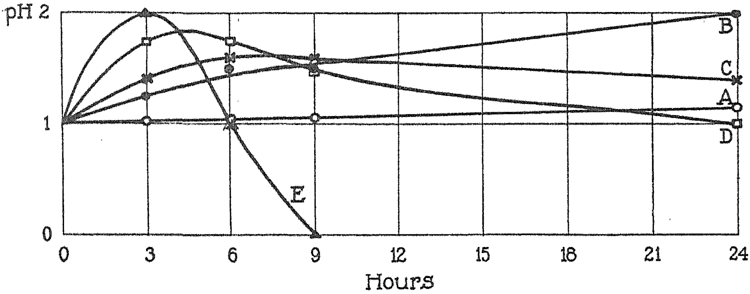

FIG. 2. Relative change of pH of solutions of alcohol in which *Elodea* had been kept. Curve A, 1 per cent; B, 1.5 per cent; C, 3 per cent; D, 6 per cent; E, 15 per cent (Table III).

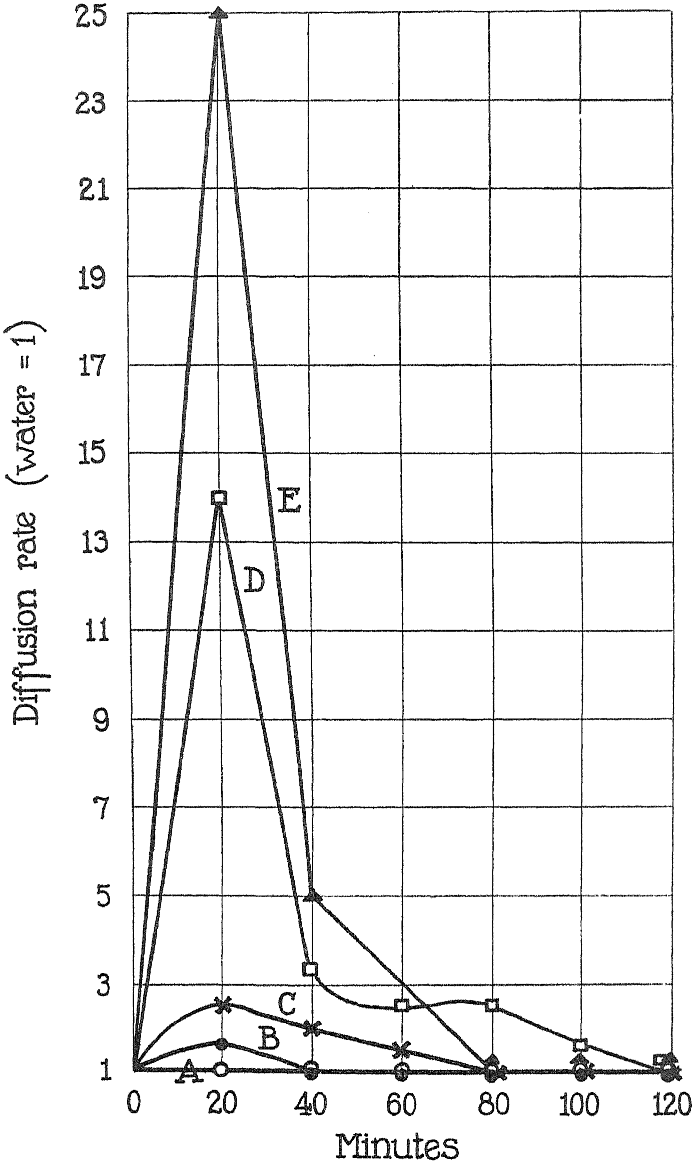

Fig. 3. Diffusion of chlorides from *Elodea* in solutions of alcohol during six 20 minute periods. Curve A, 1 per cent; B, 1.5 per cent; C, 3 per cent; D, 6 per cent; E, 15 per cent.

TABLE IV.

Rate of Diffusion of Chlorides from Elodea during Six 20 Min. Periods.

For corresponding curves, see Figs. 3, 7, 12, and 16.

Solution.	Concentration.	Diffusion by periods, 20 min. each.					
		1	2	3	4	5	6
	per cent						
Alcohol.	1.0	1.0	1.0	1.0	1.0	1.00	1.0
"	1.5	1.6	1.0	1.0	1.0	1.00	1.0
"	3.0	2.5	2.0	1.5	1.0	1.00	1.0
"	6.0	14.0	3.3	2.5	2.5	1.67	1.0
"	15.0	25.0	5.0	3.0	1.0	1.00	1.0
Ether.	1.5	1.75	1.00	1.00	1.00	1.0	1.00
"	3.0	1.67	2.50	3.33	3.33	2.0	1.67
"	5.0	20.00	3.33	2.80	2.67	2.5	2.50
"	8.0	25.00	5.00	3.00	1.00	1.0	1.00
Chloroform.	0.05	3.0	3.0	3.0	3.0	3.0	3.0
"	0.15	10.0	4.0	3.0	2.5	2.0	1.5
"	0.30	20.0	5.0	1.0	1.0	1.0	1.0
Chloretone.	0.05	1.5	1.0	1.0	1.0	1.0	1.0
"	0.10	5.0	2.0	1.0	1.0	1.0	1.0

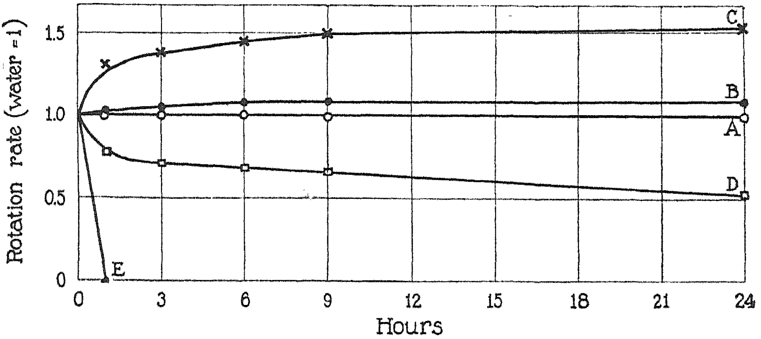

FIG. 4. Rate of protoplasmic rotation in *Elodea* in solutions of alcohol. Curve A, 1 per cent; B, 1.5 per cent; C, 3 per cent; D, 6 per cent; E, 15 per cent.

immersion for 24 hours, the rate increased still more and this more rapid rotation was continued for several days.

Since the rate of photosynthesis was normally twelve times that of respiration, the changes in rate of respiration may be omitted in determining photosynthesis without vitiating the

TABLE V.

Rate of Rotation of Elodea in Solutions of Alcohol, Ether, Chloroform, and Chloretone.

Compared with rate in water taken as the unit. For curves, see Figs. 4, 8, 13, and 17.

Solution.	Concentration.	Rotation in water.	Rate of rotation in solutions.				
			1	3	6	9	24
	per cent						
Alcohol.	1.0	1.0	0.99	1.00	1.00	1.00	0.99
“	1.5	1.0	1.02	1.05	1.07	1.08	1.09
“	3.0	1.0	1.31	1.35	1.43	1.49	1.53
“	6.0	1.0	0.76	0.71	0.67	0.67	0.52
“	15.0	1.0	0.00	0.00	0.00	0.00	0.00
Ether.	1.5	1.0	0.99	0.90	0.92	0.89	0.57
“	3.0	1.0	0.59	0.30	0.28	0.29	0.22
“	5.0	1.0	0.00	0.00	0.00	0.00	0.00
“	8.0	1.0	0.00	0.00	0.00	0.00	0.00
Chloroform.	0.05	1.0	1.17	1.30	1.31	1.34	1.36
“	0.15	1.0	0.74	0.65	0.57	0.52	0.00
“	0.30	1.0	0.00	0.00	0.00	0.00	0.00
Chloretone.	0.05	1.0	0.88	0.71	0.63	0.60	0.00
“	0.10	1.0	0.62	0.00	0.00	0.00	0.00

results (Table VI). All the solutions of alcohol produced a decrease in rate of photosynthesis, if we assume the rate of oxidation to be constant. That caused by the 1 per cent solution was so slight as to be almost within the range of possible error, but the results given are typical of those obtained in repeated experiments. No photosynthesis occurred in the 6 and 15 per cent concentrations. In these two solutions, chlorophyll diffused out of the leaves and colored the medium appreciably. Microscopic examination showed that the chloroplasts were

TABLE VI.

Photosynthesis of Elodea in Solutions of Alcohol, Ether, Chloroform, and Chloretone.

Measured in cc. of thiosulfate employed in testing the oxygen content of the solutions. Rate as compared with photosynthesis in distilled water plus $NaHCO_3$ (pH = 7.6). Immersed for two successive 1 hr. periods.

Solution.	Concentration.	Thiosulfate.	Change.	Rate of change.
	per cent	*cc.*	*cc.*	
Water.		2.25–4.28	2.03	
Alcohol.	0.05	2.25–4.20	1.95	0.96
“	0.05	2.25–4.09	0.84	0.91
Water.		2.25–4.25	2.00	
Alcohol.	1.0	2.25–3.82	1.63	0.80
“	1.0	2.25–3.70	1.45	0.73
Water.		2.25–4.25	2.00	
Alcohol.	1.5	2.25–3.66	1.41	0.69
“	1.5	2.25–3.44	1.19	0.58
Water.		2.25–4.21	1.96	
Alcohol.	3.0	2.25–3.10	0.85	0.44
“	3.0	2.25–2.25	0.00	0.00
Water.		2.25–4.25	2.00	
Alcohol.	6–15	2.25–2.25	0.00	0.00
Water.		2.25–4.25	2.00	
Ether.	1.5	2.25–3.40	1.15	0.57
“	1.5	2.25–3.20	0.95	0.42
Water.		2.25–4.24	1.99	
Ether.	3.0	2.25–2.59	0.34	0.17
“	3.0	2.25–2.25	0.00	0.00
Water.		2.25–4.37	2.12	
Chloroform.	0.05	2.25–3.35	1.10	0.52
“	0.05	2.25–3.45	1.20	0.57
Water.		2.25–4.30	2.05	
Chloretone.	0.05	2.24–4.27	2.03	1.00
“	0.05	2.24–4.30	2.06	1.00
Water.		2.25–4.33	2.18	
Chloretone.	0.1	2.24–4.32	2.08	1.00
“	0.1	2.25–4.32	2.07	1.00

considerably smaller and lighter in color. Table VII gives the sizes of the chloroplasts, computed on the basis that 1 is normal.

Ether, in all the concentrations employed, caused an increase in the rate of respiration (Table I and Fig. 5), but the 8 per cent solution produced less initial increase than any of the others.

TABLE VII.

Size of Chloroplasts.

Measured by means of camera lucida drawings after the plant had been immersed for 3 hrs. in solutions of alcohol, ether, chloroform, and chloretone.

Solution.							
Water.	Size (average control)	6.61	6.59	6.59	6.00		
Alcohol.	Concentration, per cent	**0.05**	**1.00**	**1.50**	**3.00**	**5.00**	**15.00**
	Size*	6.67	6.62	6.57	6.26	3.42	2.91
	Ratio to normal	1.00	1.00	0.99	0.79	0.52	0.44
Ether.	Concentration, per cent	**1.50**	**3.00**	**5.00**	**8.00**		
	Size*	5.02	4.50	3.71	2.32		
	Ratio to normal	0.76	0.68	0.56	0.36		
Chloroform.	Concentration, per cent	**0.05**	**0.15**	**0.30**			
	Size*	6.03	3.58	2.47			
	Ratio to normal	0.91	0.54	0.37			
Chloretone.	Concentration, per cent	**0.05**	**0.10**				
	Size*	6.61	6.58				
	Ratio to normal	1.00	0.99				

*Size indicated in units on micrometer scale.

In this anesthetic, as in alcohol, the greatest change in the pH of the medium was caused by the most concentrated solution (8 per cent, Table III and Fig. 6). In both the 5 and 8 per cent solutions chlorides diffused from the plant with such rapidity as to indicate that the cells must be injured (Table IV and Fig. 7). Microscopic examination showed that protoplasmic rotation had stopped completely in the 5 per cent solution at the end of 20 minutes, and in the 8 per cent solution at the end of 10 minutes (Table V and Fig. 8). Both were irreversible. In the 8 per cent solution the cells were plasmolyzed (false plasmolysis), and after 24 hours immersion disintegration of the cell structures had set in. In the 5 per cent solution most of the cells were plasmolyzed after 2 hours. The relative size of the chloroplasts after 3

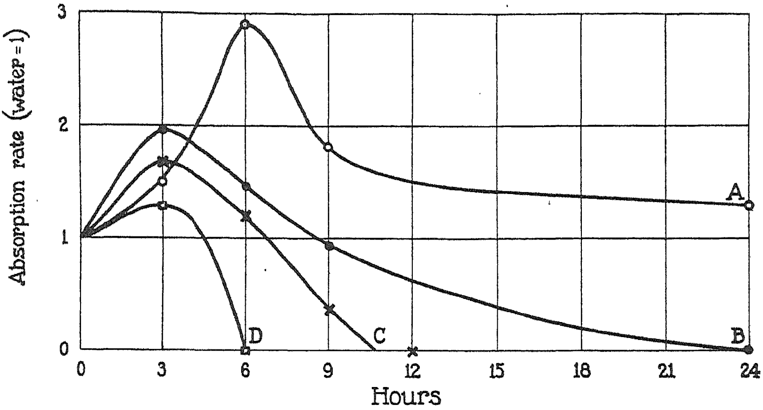

FIG. 5. Relative rate of O_2 absorption of *Elodea* in solutions of ether. Curve A, 1.5 per cent; B, 3 per cent; C, 5 per cent; D, 8 per cent.

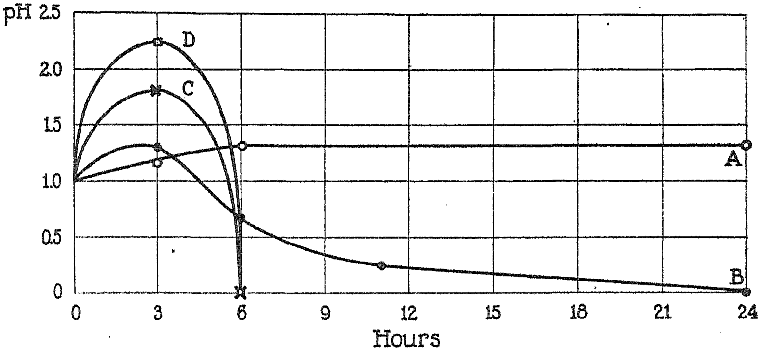

FIG. 6. Relative change of pH of solutions of ether in which *Elodea* had been kept for 3 hour periods. Curve A, 1.5 per cent; B, 3 per cent; C, 5 per cent; D, 8 per cent.

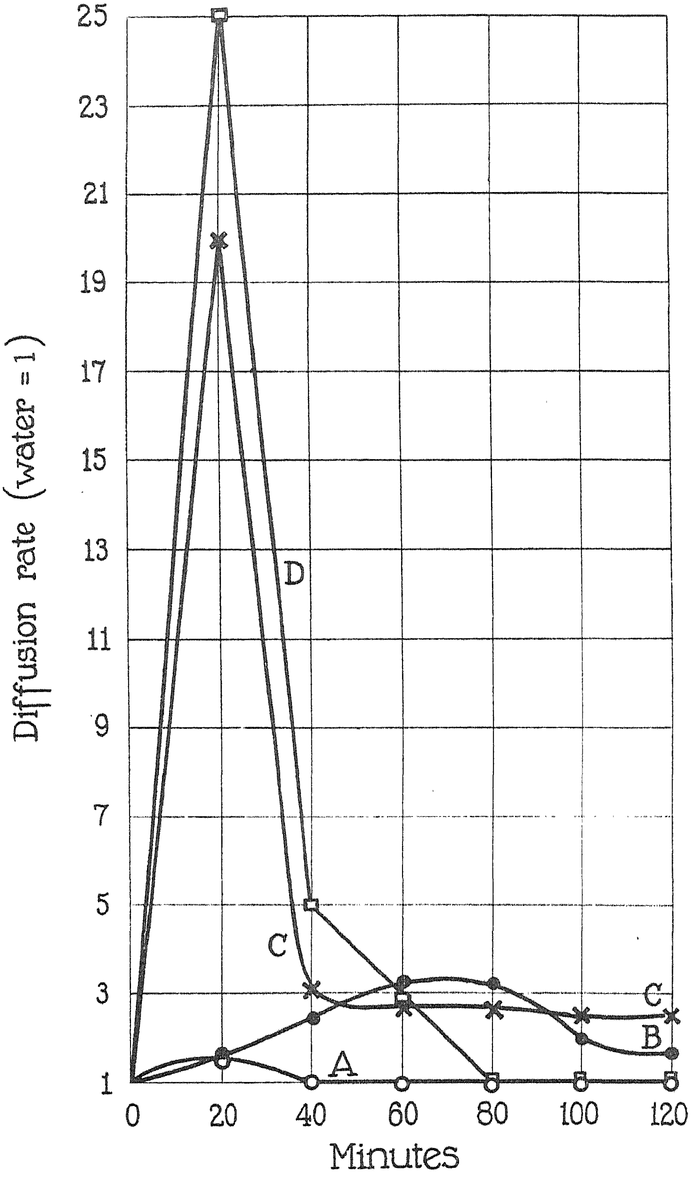

Fig. 7. Diffusion of chlorides from *Elodea* in solutions of ether during six 20 minute periods. Curve A, 1.5 per cent; B, 3 per cent; C, 5 per cent; D, 8 per cent.

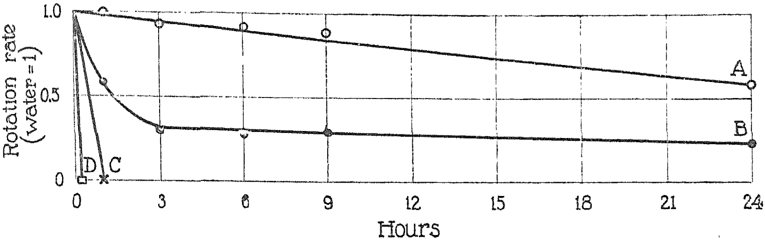

FIG. 8. Rate of protoplasmic rotation in *Elodea* in solutions of ether. Curve A, 1.5 per cent; B, 3 per cent; C, 5 per cent; D, 8 per cent.

hours in the various concentrations of ether is shown in Fig. 9, Nos. 2 to 5.

In the experiments with chloroform the differences between rates of respiration as determined by O_2 consumption and by change of pH are similar to those which occurred in alcohol. In the most concentrated solution (0.3 per cent) the greatest change in pH occurred, while O_2 consumption was less than that in the

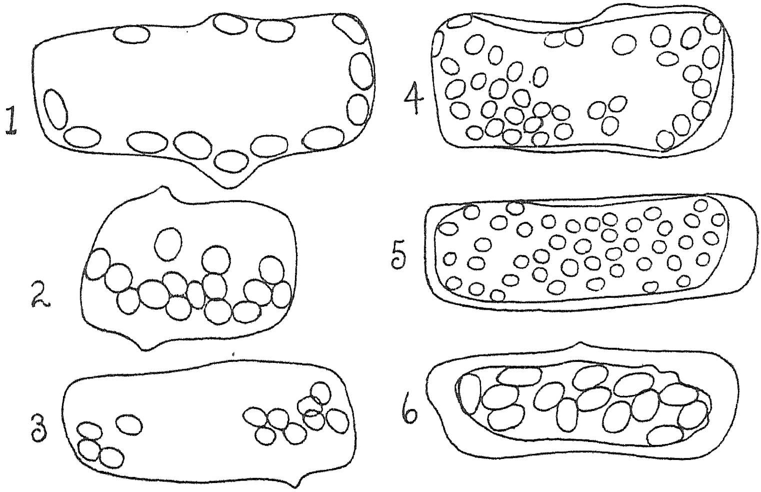

FIG. 9. Camera lucida drawing of *Elodea* cells. No. 1, normal cell; No. 2, cell exposed 2 hours to 1.5 per cent ether; No. 3, cell exposed 2 hours to 3 per cent ether; No. 4, cell exposed 2 hours to 5 per cent ether; No. 5, cell exposed 2 hours to 8 per cent ether; No. 6, cell exposed 24 hours to 0.1 per cent chloretone.

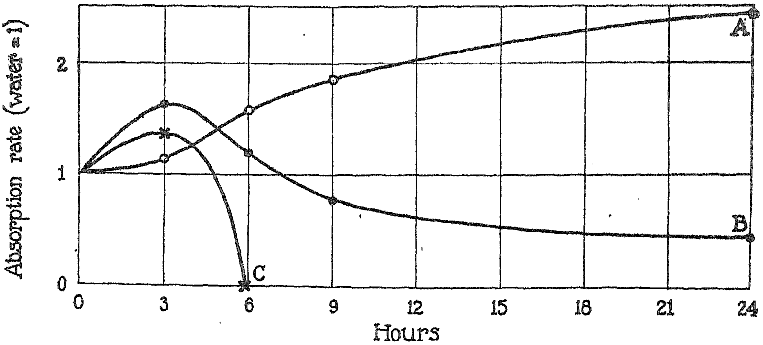

FIG. 10. Rate of O_2 absorption of *Elodea* in solutions of chloroform. Curve A, 0.05 per cent; B, 0.15 per cent; C, 0.30 per cent (Table I).

1.5 per cent solution (Tables I and III and Figs. 10 and 11). Rate of diffusion of chlorides from the cell was increased in all the solutions (Table IV and Fig. 12), but in none of them did it reach as high a figure as with alcohol or ether. In the 0.3 per cent solution exosmosis practically stopped after immersion for 40 minutes, showing that diffusion does not continue very long after the death of the cell.

Rotation stopped after immersion for 2 minutes in 0.3 per cent chloroform (Fig. 13, Curve C), but when the plant was returned

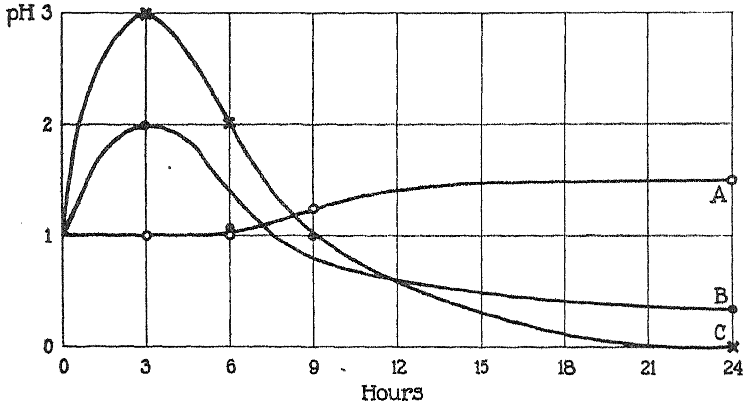

FIG. 11. Relative change of pH of solutions of chloroform in which *Elodea* had been immersed for 3 hour periods. Curve A, 0.05 per cent; B, 0.15 per cent; C, 0.30 per cent.

to water it reappeared at the end of 2 minutes. After 4 minutes in 0.3 per cent chloroform it required 45 minutes for rotation to return to normal, and after being immersed for 10 minutes the cells were irreversibly plasmolyzed and the pigment was largely

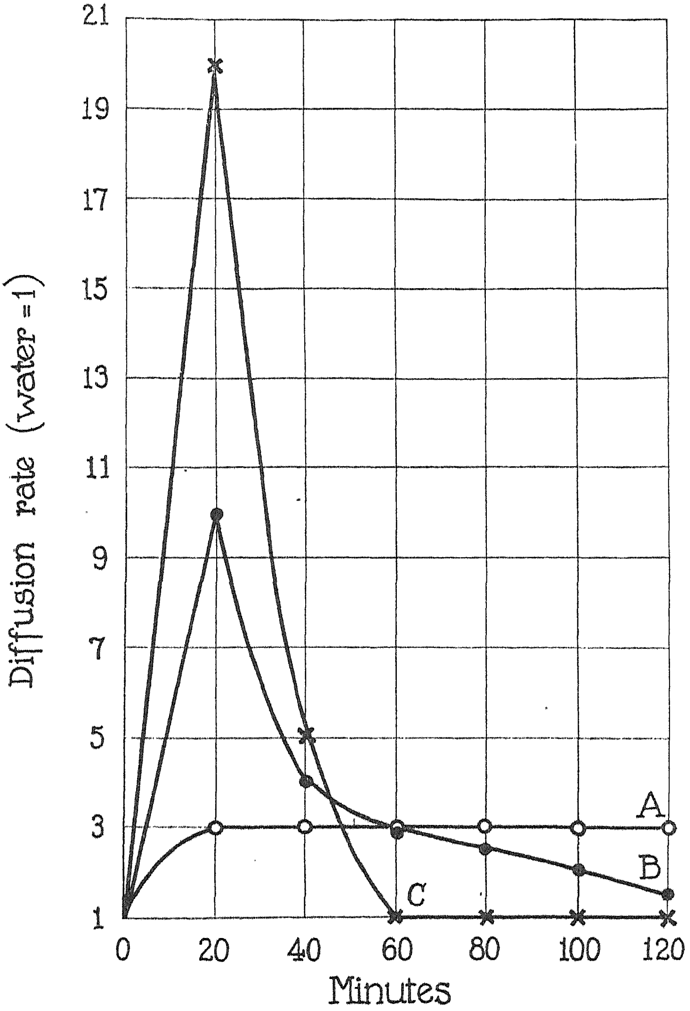

FIG. 12. Diffusion of chlorides from *Elodea* in solutions of chloroform for six 20 minute periods. Curve A, 0.05 per cent; B, 0.15 per cent; C, 0.30 per cent.

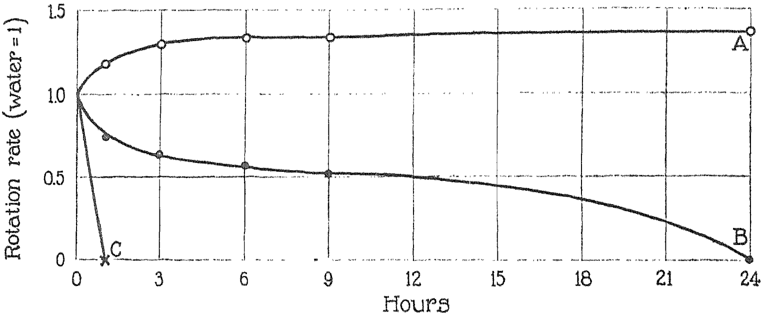

FIG. 13. Rate of protoplasmic rotation in *Elodea* in solutions of chloroform. Curve A, 0.05 per cent; B, 0.15 per cent; C, 0.30 per cent.

extracted from the chloroplasts. In both 0.15 (Curve B) and 0.3 per cent solutions of chloroform no photosynthesis occurred. In the latter, after 10 minutes immersion, photosynthesis did not reappear when the plant was returned to tap water, but in the 0.15 per cent solution photosynthesis reappeared even after 9 hours immersion.

In 0.1 per cent chloretone (Fig. 14, Curve B) respiration, measured by O_2 consumption, decreased immediately to a very low level, less than 25 per cent of the normal, but was completely reversible, resuming its original rate within 30 minutes after

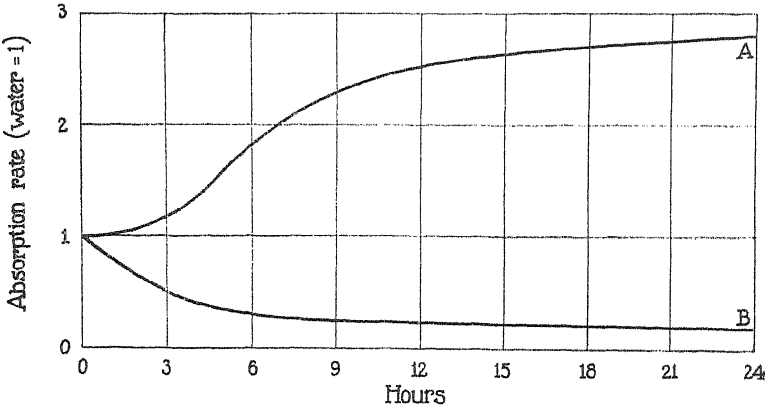

FIG. 14. Relative rate of O_2 absorption by *Elodea*. Curve A, 0.05 per cent chloretone; B, 0.1 per cent chloretone.

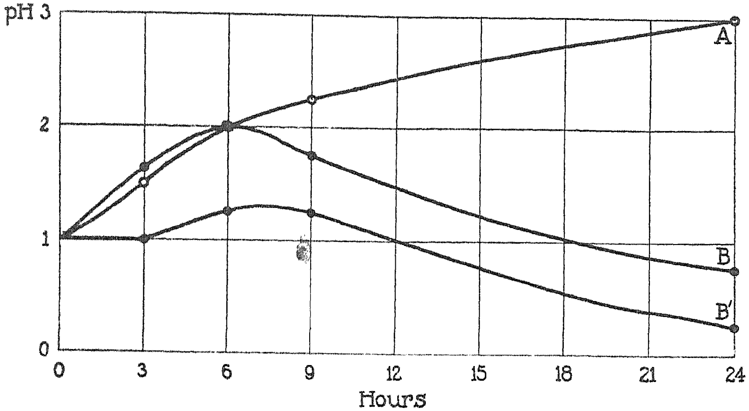

FIG. 15. Relative change of pH of solutions of chloretone during 3 hour periods. Curve A, 0.05 per cent; B, 0.1 per cent; B′ is from another experiment with plant not growing rapidly.

being returned to tap water. CO_2 production in 0.1 per cent chloretone (Fig. 15, Curve B) increased at first and then gradually fell below the normal. When tips of *Elodea* were growing less rapidly, the acceleration was much less marked, and the level reached after 24 hours considerably lower, as shown in Fig. 15, Curve B′, where it was 25 per cent of the normal. Even in this case, however, the condition was reversible. In cases of still less vigorous stalks, no acceleration took place, and these also were apparently uninjured by the anesthetic. Table VIII gives an example.

After 24 hours in 0.1 per cent chloretone the cells usually were more or less plasmolyzed (Fig. 9, No. 6). The chloroplasts, although of normal size, showed no rotation and did not change

TABLE VIII.

Time.	Reaction in water.	Reaction in 0.1 per cent chloretone solution
min.	*pH*	*pH*
0	7.80	7.65
30	7.75	7.65
60	7.70	7.60
120	7.60	7.55
180	7.50	7.45
360	7.20	7.40

position when the plant was placed in strong sunlight. Within 1 hour after returning to tap water, however, all trace of anesthesia disappeared. Fig. 16 shows exosmosis of chlorides, and Fig. 17 rate of protoplasmic rotation, under the influence of the chloretone solutions.

In Table IX are tabulated the ratios of the various cell activities. Figures for O_2 consumption, change in pH by CO_2 production, protoplasmic rotation, and size of chloroplasts are taken from Tables I, III, V, and VII, respectively. Time of immersion in each case was 3 hours. Since the data in Tables IV and VI on photosynthesis and diffusion of chlorides were obtained after shorter intervals, the figures presented here have been taken from other experiments in which the period of treatment was for 3 hours.

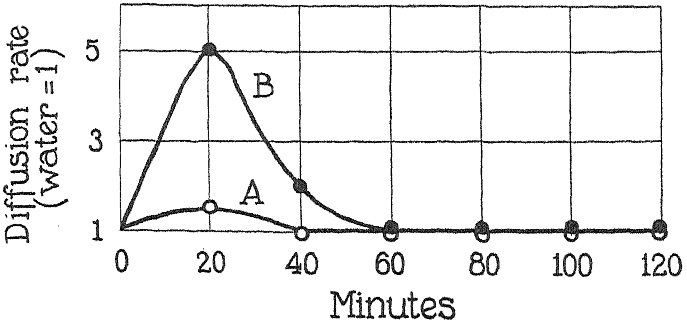

FIG. 16. Diffusion of chlorides during six 20 minute periods. Curve A, 0.05 per cent; B, 0.1 per cent chloretone.

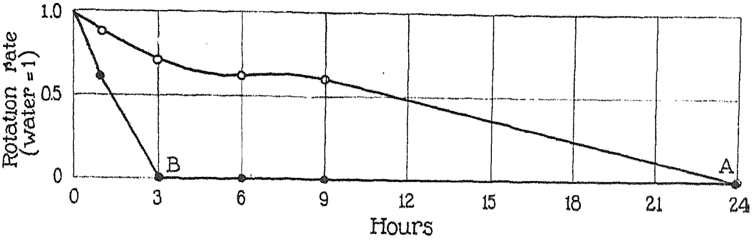

FIG. 17. Rate of protoplasmic rotation in solutions of chloretone. Curve A, 0.05 per cent; B, 0.1 per cent.

The only solutions which did not cause increase in rate of respiration, as measured by the O_2 consumption, were 15 per cent alcohol and 0.1 per cent chloretone. In the former the plant was killed almost instantly, but in the latter the inhibition was completely reversible. Hence no general rule seems to hold for all the anesthetics employed. In all cases the change of hydrogen ion concentration due to CO_2 production increased in higher concentrations of the anesthetic, and was greatest in the cells that were injured beyond recovery. In the 6 per cent alcohol,

TABLE IX.

Ratios of the Rate of the Various Cell Processes, during 3 Hrs. Anesthesia, to the Normal Condition Taken as the Standard, and Expressed as 1.

Figures under "Photosynthesis" and "Diffusion" taken from data not presented in the tables. Other data from Tables I, III, V, and VII.

Solution.	Concentration.	O_2 consumption.	pH	Rotation.	Photosynthesis.	Size of chloroplasts.	Diffusion.	Irreversible changes.
	per cent							
Water.		1	1	1	1	1	1	
Alcohol.	0.50				0.94	1.00	1.0	
	1.00	1.12	1.00+	1.00	0.75	1.00	1.0	
	1.50	1.35	1.25	1.05	0.62	0.99	1.5	
	3.00	1.89	1.40	1.35	0.44	0.79	5.0	
	6.00	1.63	1.75	0.71	0.00	0.52	20.0	
	15.00	0.83	2.00	0.00	0.00	0.44	35.0	Plasmolysis.
Ether.	1.50	1.50	1.17	0.90	0.49	0.76	2.0	
	3.00	1.86	1.33	0.30	0.12	0.68	10.0	
	5.00	1.69	1.83	0.00	0.00	0.56	30.0	Rotation stopped.
	8.00	1.29	2.25	0.00	0.00	0.36	35.0	Plasmolysis.
Chloroform.	0.05	1.13	1.00	1.30	0.47	0.91	15.0	
	0.15	1.63	2.00	0.00	0.00	0.54	20.0	Plasmolysis. Photosynthesis stopped.
	0.30	1.38	3.00	0.00	0.00	0.37	25.0	
Chloretone.	0.05	1.17	1.50	0.71	1.00	1.00	1.5	
	0.10	0.50	1.62	0.00	1.00	0.99	7.5	

change of pH was increased to 1.75 times the normal for the first 6 hours (Table III). These cells showed some signs of injury; when the tip of *Elodea* was not very vigorous, the leaves were killed, and even in rapidly growing tips some of the less vigorous cells were irreversibly plasmolyzed. The 5 per cent ether caused a change of pH 1.83 times that of the control by the end of the first 3 hours, and the cells were injured beyond recovery. The 0.1 per cent chloretone caused only a slightly greater CO_2 production, as measured by the change in pH, than did the 0.05 per cent solution. The cells were markedly affected by the anesthetic, many were plasmolyzed, in all of them rotation had stopped, and the chloroplasts did not change position when the plants were placed in the sunlight.

In each anesthetic, the solution of greatest concentration caused the greatest change in pH, whereas the amount of oxygen used increased through the lower concentrations of the anesthetic, reached a maximum in the solution just failing to cause permanent injury to the cell, and then decreased in those causing irreversible changes. Such an effect indicates that in respiration at least two separate processes must be involved. It is possible that the cells in strong concentrations of anesthetics more or less completely lose their power to take up oxygen (due to injury).

The 0.1 per cent solution of chloretone was the only one in which rotation was completely stopped before the cell was irreversibly injured. In all cases, however, the leaf cells seemed to react to the anesthetic much more readily than the stem cells, so that there was considerable variation as to this point even in a single leaf. In the 3 per cent ether and the 0.15 per cent chloroform, for instance, most of the movement was confined to the cells of the midrib. The leaf cells, however, recovered upon being returned to tap water.

Photosynthesis seems to be the most readily affected of any of the cell processes by alcohol, ether, and chloroform; but as these are all well known solvents of chlorophyll, such a result is probably to be expected. Decrease of size of the chloroplasts was increasingly evident in the more concentrated solutions of the three anesthetics, and it may be significant that in each case photosynthesis stopped when the chloroplasts became reduced to about one-half their normal size. In the 5 per cent ether the cells were killed; but in the 6 per cent alcohol and the 0.05 per cent chloroform the change was reversible.

Diffusion of chlorides from the cell was enormously increased in all those cases where the plant was injured beyond recovery; *e.g.*, in 15 per cent alcohol, thirty-five times that in water; in 5 per cent ether, thirty times that in water; in 8 per cent ether, thirty-five times that in water; and in 0.3 per cent chloroform, twenty-five times that in water. Both 6 per cent alcohol and 0.15 per cent chloroform caused diffusion of chlorides twenty times that of the control. These two solutions seemed to produce about the same degree of change in all the cell processes and to leave the cell afterwards in a very similar condition. In those cases where rate of diffusion was ten times the normal or less, no permanent injury resulted.

Experiments on the Marine Jellyfish, Cassiopea.

In determining the rate of respiration, four jellyfish with manubria and nerve centers removed (15 cm. in diameter) were placed in sea water in a tightly sealed jar of 1 liter capacity and rotated in a thermostat for 1 hour in the total absence of light (to prevent photosynthesis by symbiotic plant cells). Control of temperature and O_2 content of the medium from the moment of starting the experiment was accomplished in the following manner. The thermostat was very large and was filled with fresh sea water heated quickly to the temperature of 30° by a special device (the sea being at 28–29°). The pH was rapidly determined colorimetrically and the jellyfish and jar were placed in the thermostat. The bottle containing the sample of water was sunk in the thermostat, the jellyfish were quickly placed in the jar, and the rotation was started. The water sample bottle was removed and its contents were titrated with thiosulfate, after which it was filled with pure mercury, and a two-hole rubber stopper with two tubes was inserted in place of the glass stopper. At the end of the hour the jar was opened and one of the tubes from the sample bottle inserted to the bottom. On inverting the bottle, the mercury ran out of one tube and thus sucked in water from the bottom of the jar through the other, without gaseous exchange with air. Since the jellyfish remained in the jar during the operation, and respiration continued, extreme rapidity had to be employed in filling the bottle. The sample for pH determination was merely dipped out of the jar in a beaker, as it was found that any change that might occur through momentary contact with air was incapable of being measured under these conditions, in which the difference of CO_2 tension in the water and air was a small fraction of a millimeter of mercury.

Owing to the fact that the Tortugas Laboratory closed unusually early, the experiments were confined to the effects of ether and of CO_2. It was found that the neuromuscular system of the umbrella of *Cassiopea* was prevented from responding to electrical stimuli by the addition of 0.5 per cent or more ether to the medium. The respiratory quotient was found to be about 0.95, and, since the oxygen data are more accurate than the CO_2 determinations, only the former are given. In no case was the O_2 concentration at the end of an experiment reduced more than 50 per cent in the medium (5 cc. per liter at the beginning of experiment and more than 2.5 cc. at the end).

It had previously been shown that the rate of O_2 consumption depended on O_2 concentration,[1] and therefore the attempt was made to have the O_2 concentration the same at the beginning of all experiments. This was not always realized, however. Ether was mixed with the sea water in two 10 liter glass-stoppered bottles that were filled so completely as to exclude air bubbles. As the ether dissolved, a vacuum was produced and air bubbled out of the water to fill this space. The O_2 content of the water was reduced 25 per cent (or less), which, according to previous experiments, should decrease O_2 consumption 10 per cent (or less). Since 0.1° error in temperature makes a change in O_2 consumption of about 2.5 per cent and the error in titration may be 3 per cent, and since a small error occurs in taking the water samples, the probable error of an experiment may be nearly 10 per cent. It would be superfluous, therefore, to apply corrections of less than 10 per cent for decreased O_2 tension. In Table X the figures in bold-faced type have been raised 10 per cent to correct for 25

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[1] McClendon, J. F., *J. Biol. Chem.*, 1917, xxxii, 291 (table). The statements to the contrary in summaries by the same author (McClendon, J. F., *J. Biol. Chem.*, 1920, xli, p. lxiv; *Year Book of the Carnegie Inst. Washington*, 1919, xviii, 203) are erroneous.

TABLE X.

Percentage of ether	0.5	1.0	1.0	1.0	2.0	2.0	3.0	4.0
O_2 used (control), *cc*	2.4	2.4	1.8	2.7	2.2	2.1	1.2	2.1
O_2 " in ether, *cc*	2.4	2.4	1.8	2.7	2.0	2.2	1.2	1.3
Ratio with control	1.0	1.0	1.0	1.0	0.9	1.05	1.0	0.6

per cent lowering in O_2 concentration; the pH was 8.2 at the beginning of each experiment.

The animal died in 4 per cent ether and hence the time of respiration was less than in the control and the experiment must be disregarded. In the remaining experiments, only one (in 2 per cent ether) shows a variation from the control of as much as 10 per cent. Since 10 per cent seems to be within the limit of error, the experiments do not show a change of O_2 consumption during anesthesia with ether.

CO_2 has been used to anesthetize marine animals and seems to be more effective than ether, as the latter stimulates certain cells as evidenced by the discharge of mucus. CO_2 was added to sea water in a Sparklet fountain and mixtures were made of 10, 20, 30, 40, and 50 per cent of this carbonated water with water taken directly from the sea. A correction should be made for lowered O_2 content of the carbonated water, as is done in Table XI under "corrected data."

TABLE XI.

pH	6.6	6.3	5.8	5.7	5.5
Total CO_2 per liter, *cc*	68	73	80	82	85
O_2 used in carbonated water, *cc*	2.0	1.9	1.6	0.7	0.4
O_2 " " " " (corrected data), *cc*	2.0	1.9	1.8	1.0	0.5
O_2 used in control ($pH = 8.2$, $CO_2 = 44$), *cc*	2.1	2.1	2.6	1.7	1.7
Ratio to control	0.95	0.90	0.69	0.59	0.29

It may be seen from Table XI that the progressive decrease in O_2 consumption with increase in CO_2 is certainly far beyond the limit of error of the method. The addition of the gas to sea water increases the concentration of CO_2 molecules, HCO_3 ions, and H ions. In the experiments recorded in Table XII, HCl was added to sea water to determine whether the H ions were entirely responsible for the decreased O_2 consumption. The O_2 content was not altered.

TABLE XII.

pH after adding HCl	6.6	5.8
O_2 used after HCl was added, *cc*	2.0	1.3
O_2 " in control (pH = 8.2), *cc*	2.1	1.9
Ratio to control	0.95	0.69

It may be seen that the difference between the action of CO_2 gas (Table XI) and HCl which liberates CO_2 from carbonates (Table XII) is *nil* or at least far within the limit of error of the experiments. The experiments recorded in Table XIII were made by adding enough HCl to neutralize exactly the alkaline reserve, after which we boiled off the CO_2 and increased the H ion concentration by addition of traces of phosphoric acid (not sufficient to be toxic).

TABLE XIII.

O_2 used at pH = 8.2 (control), *cc*	2.9
O_2 " " pH = 5.8 (CO_2-poor), *cc*	2.7
Ratio to control	0.93

It may be inferred from Table XIII that by changing the pH to 5.8 without increase of CO_2 respiration was not depressed (93 per cent of the control being within the limit of error of the method). This experiment was performed with the most extreme care since it was evident that time would not be available for a repetition of it. It indicates that H ions are not the only factor in depressing respiration, and the CO_2 molecules or ions are depressants. Perhaps the CO_2 inhibits respiration by acting as the end-product in a reversible reaction. The fact that respiration in *Cassiopea* varies directly with O_2 concentration indicates that oxidation is a simpler phenomenon in this animal than in those organisms in which CO_2 production is more or less independent of O_2 consumption.

STUDIES IN NUTRITION.

IV. THE NUTRITIVE VALUE OF PEANUT FLOUR AS A SUPPLEMENT TO WHEAT FLOUR.

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(Received for publication, May 28, 1920.)

Valuable studies on the milling products of wheat have been made by Osborne and Mendel (1) who have shown that commercial patent wheat flour is deficient in water-soluble vitamine as well as in the quality of its protein mixture. Voegtlin, Lake, and Myers (2) have also shown that "patent" and "highly milled" wheat flours are deficient in water-soluble vitamine and recommend the use of whole wheat flours.

As will be shown later, bread made from "war flour" which contained 74 per cent of the wheat kernel furnished sufficient water-soluble vitamine for normal growth of albino rats provided the other essentials of a complete diet were supplied. However, bread made from "war flour" lacks an efficient mixture of proteins; even when supplemented with the other non-protein ingredients of a complete diet it enables albino rats to grow at only one-third to two-thirds of the normal rate. Bread made from patent flour which is more commonly used is a still poorer food.

We have found that peanut flour is an efficient and palatable supplement to wheat flour. Peanut flour is made by grinding the press-cake which is obtained as a by-product when shelled peanuts are pressed to produce peanut oil. Such a flour contains about 7 per cent of fat and 50 per cent of proteins. The protein content of the peanut flour is therefore from four to five times as high as in wheat flour. Peanut press-cake has been used as a cattle feed and to some extent as a fertilizer because its great value as a human food has not been generally understood. The value of the proteins of the peanut have been demonstrated by chemical work (3, 4) and by nutrition experiments performed by Daniels and Loughlin (5), Osborne and Mendel,[1] and the writers.[2] Holmes (6) has also shown that the proteins, fats, and carbohydrates of peanut flour are easily digested by human subjects. Very palatable bread can be made from wheat flour to which from 15 to 25 per cent of peanut flour has been added. Such bread is rich in water-soluble vitamine and contains a protein mixture adequate for normal growth at a cost of less than one-fifth of the cost of proteins derived from animal sources.

A bread has also been prepared which contains the proteins, salts, and vitamines needed for the normal growth of albino rats. With this it has been found possible to obtain normal growth on a diet of only bread and water.

Wheat Flour Used in Experiments.—Two types of wheat flour were used to prepare the breads described herein. One was a "war flour" containing about 74 per cent of the wheat kernel and this will be designated as Flour A. This contained 0.56 per cent of ash and 2.01 per cent of nitrogen. The second type, designated as Flour B, was especially milled for another project. It was made from Kansas hard wheat and was assembled from mill stream middlings Nos. 1, 2, 3, 4, 5, and one-half of No. 6 and sizing stocks. This flour contained 0.55 per cent of ash and 2.11 per cent of nitrogen.

Bread Used in Experiments.—Breads were made from the wheat flour alone and from mixtures of the wheat flours and peanut flour. Bread made from 25 parts of peanut flour and 75 parts of wheat flour will be designated as 25 per cent peanut bread, while that made from 15 parts of peanut flour and 85 parts of wheat flour will be called 15 per cent peanut bread.

The breads[3] were made according to the following formula.

	gm.
Flour	400
Compressed yeast	10
Granulated sugar	10
Table salt	6
Water	228

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[1] Private communication.

[2] Unpublished data.

[3] The bread used in this experiment was made by Miss Pearl A. Bernhardt of the Protein Investigation Laboratory, Bureau of Chemistry.

The above quantities were used for one loaf and ten such loaves were baked each time. The salt and sugar were dissolved in the water and the solution was mixed with the yeast. This suspension was then mixed thoroughly with the flour and the resulting dough was handled in the usual manner. The peanut breads were made in the same way using the proper proportions of wheat and peanut flour and mixing these carefully. After baking, the bread was sliced, dried at 60°C., and ground to a powder which was used for preparing the diets.

Calculation of the Protein Content of the Breads.—The percentage of protein in the bread was calculated by multiplying the percentage of nitrogen found by 5.7. This is the factor used for gliadin in wheat and is also nearly the factor for converting nitrogen to protein in the peanut proteins (3).

Growth Obtained on Wheat Bread.—Experiments were performed with bread made wholly from either Flour A or B as previously described. The diets were composed of 80 parts of dried bread, 16 parts of butter fat, and 4 parts of a salt mixture.[4] The rats fed on the diet containing bread from Flour A grew approximately one-third to two-thirds of the normal rate. Of six rats used four were kept on this diet for from 270 to 315 days when the experiment was discontinued. One of the other rats began to decline after growing for about 160 days. Another one died at the end of 160 days. The growth curves are shown in Charts 1 and 2.

The failure of the rats to grow normally was not due to a deficit of water-soluble vitamine since normal growth was obtained when 12 per cent of purified casein replaced an equivalent amount of bread in the diet. Since yeast contains water-soluble

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[4] The composition of the salt mixture used in rations described herein is the same as that used by Osborne and Mendel in their experimental diets. See Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 374.

	gm.		gm.
$CaCO_3$	134.8	Citric acid + H_2O	111.1
$MgCO_3$	24.2	Fe citrate + $1\frac{1}{2}$ H_2O	6.34
Na_2CO_3	34.2	KI	0.020
K_2CO_3	141.3	$MnSO_4$	0.079
H_3PO_4	103.2	NaF	0.248
HCl	53.4	$K_2Al_2(SO_4)_4$	0.0245
H_2SO_4	9.2		

vitamine none was incorporated in the bread used for this experiment. The growth curves obtained are shown in Chart 3.

Growth Obtained on 25 Per Cent Peanut Bread.—Two different diets were prepared from this bread, one containing from 16.3 to

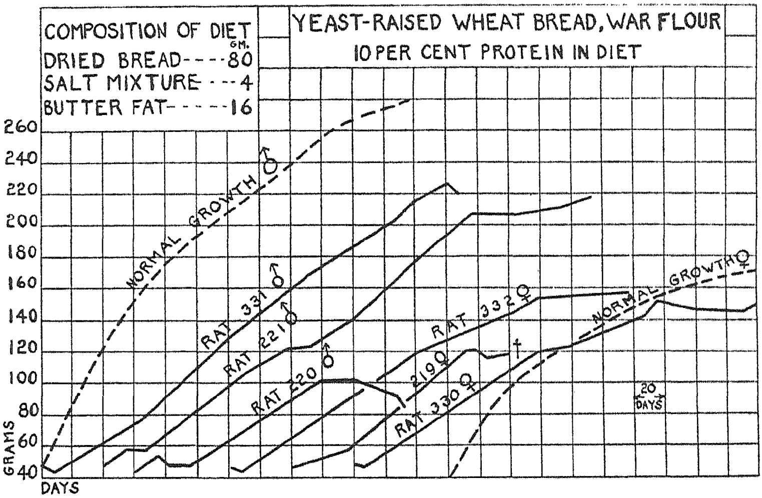

CHART 1.

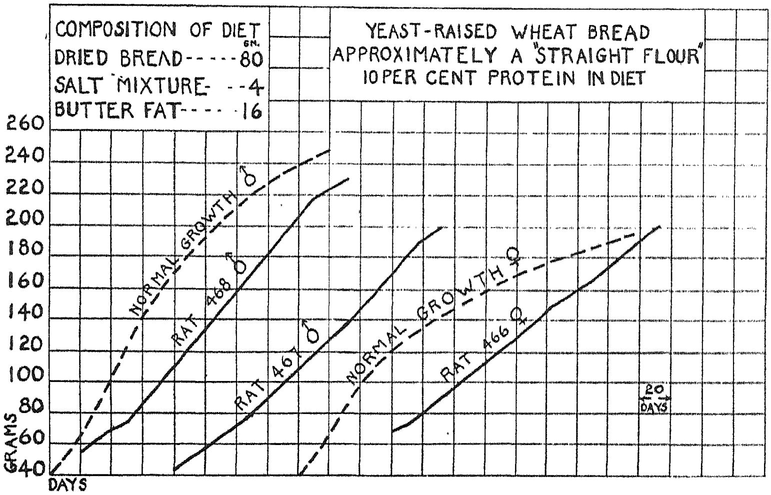

CHART 2.

16.8 per cent and the other 10 per cent of protein. The rats fed on the diets containing 16.3 or 16.8 per cent of protein grew normally. The composition of the diet and the growth curves

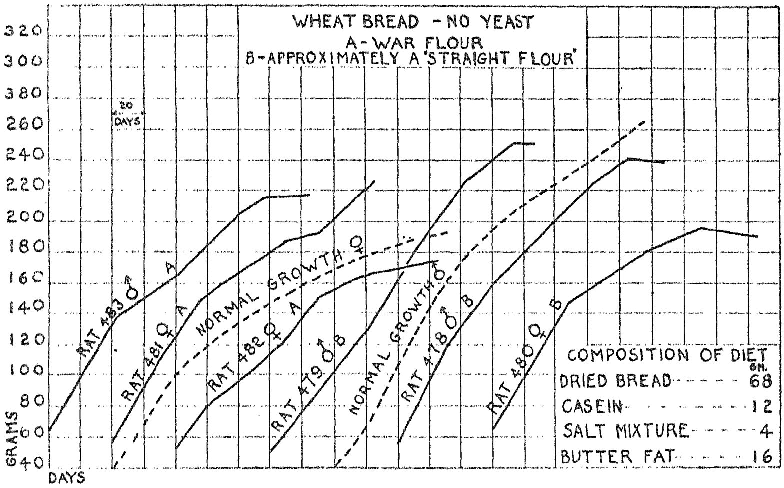

CHART 3.

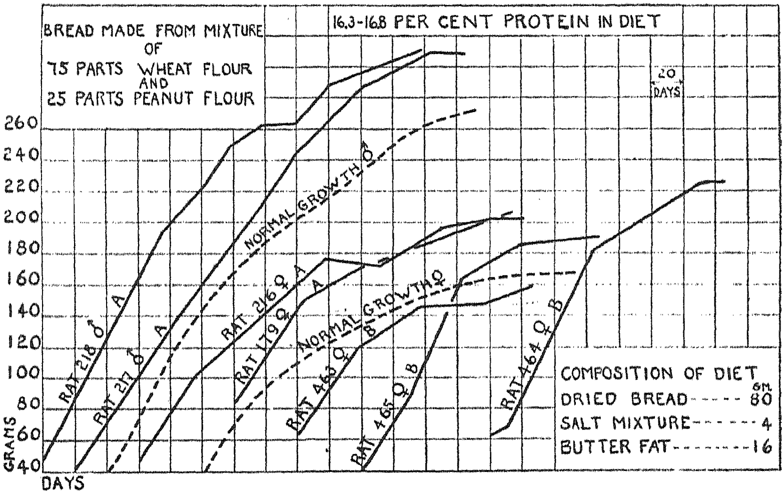

CHART 4.

are shown in Chart 4. The diet containing 10 per cent of protein was prepared by replacing a part of the bread with starch. This also produced normal growth but with a slight retardation during the first few weeks. The results are shown in Chart 5.

The excellent growth obtained on this diet containing but 10 per cent of proteins derived from bread made with a mixture of peanut flour and wheat flour in contrast with the poorer growth on a wheat bread diet which also contained 10 per cent of proteins, shows clearly the superiority of the peanut bread.

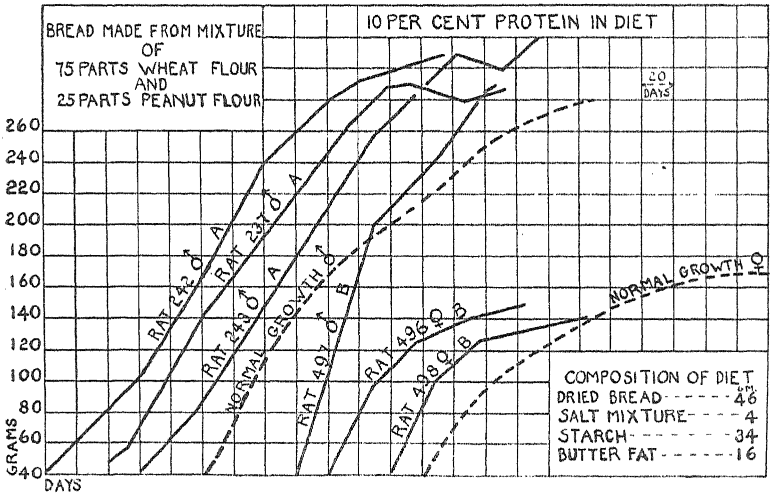

CHART 5.

Growth Obtained on 15 Per Cent Peanut Bread.—A diet was prepared from this bread and contained 14 per cent of protein. The rats fed on this diet grew at almost a normal rate as shown by the curves in Chart 6.

Efficiency of the Proteins in the Different Breads.—During the course of these experiments the food intakes were recorded in order to establish index numbers that would give the ratio of the gains in weight to the grams of protein ingested.

In doing this we have followed the procedure suggested by Osborne, Mendel, and Ferry (7). It is to be understood that the ratios obtained by us give only approximate results. The diets varied somewhat in calorific value which influenced the quantity of food ingested. The rates at which different rats grow vary considerably and more accurate results could have been obtained by using more animals in each of the experiments. We think, however, that the comparative data given in Tables I and II show that a mixture of wheat and peanut flour furnishes more efficient proteins than those found in wheat flour alone. It is also apparent that the proteins of the peanut breads are much better utilized than those in the wheat bread.

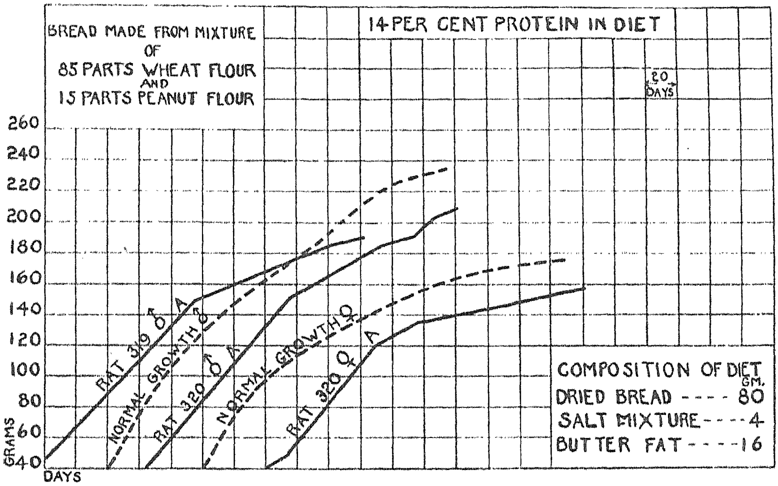

Chart 6.

SUMMARY.

1. A diet containing bread made from wheat flour (74 per cent extraction) when fed to albino rats as the only source of protein and water-soluble vitamine, together with an adequate inorganic salt mixture and butter fat, produced only about one-third to two-thirds of normal growth.

2. Bread made with a mixture of 25 parts of peanut flour and 75 parts of wheat flour furnished adequate proteins and water-soluble vitamine for normal growth. A similar bread containing 15 parts of peanut flour and 85 parts of wheat flour contained proteins and sufficient water-soluble vitamine for growth at very nearly the normal rate.

TABLE I.

Gain of Body Weight Per Gm. of Ingested Protein Furnished by 15 and 25 Per Cent Peanut Bread and Wheat Bread (War Flour).

Source of protein.	Protein in food.			Rat.	Initial body weight.	Gain in 4 wks.	Gain in 10 wks.	4 wks. period.					10 wks. period.				
								Total intake.		Intake per gm. of gain.		Gain per gm. of protein.	Total intake.		Intake per gm. of gain.		Gain per gm. of protein.
	From wheat flour.	From peanut flour.	Total protein in diet.					Food.	Protein.	Food.	Protein.		Food.	Protein.	Food.	Protein.	
	per cent	*per cent*	*per cent*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
25 per cent peanut bread.	6.8	9.5	16.3	216♀	47	48	95	205	33.4	4.3	0.70	1.44	545	88.8	5.73	0.93	1.07
				217♂	42	38	108	175	28.5	4.6	0.75	1.33	498	81.2	4.62	0.75	1.33
				218♂	48	57	138	213	34.7	3.7	0.61	1.64	614	100.1	4.45	0.73	1.38
Average										4.2	0.69	**1.47**			4.93	0.80	**1.26**
25 per cent peanut bread.	4.2	5.8	10.0	242♂	42	27	84	218	21.8	8.1	0.81	1.24	582	58.2	6.93	0.69	1.44
				243♂	43	28	99	200	20.0	7.1	0.71	1.40	597	59.7	6.03	0.60	1.66
				237♂	48	30	100	214	21.4	7.1	0.71	1.40	673	67.3	6.73	0.67	1.48
Average										7.4	0.74	**1.35**			6.56	0.65	**1.53**
15 per cent peanut bread.	8.1	5.9	14.0	318♀	42	28	82	153	21.4	5.5	0.76	1.31	473	66.2	5.77	0.81	1.24
				319♂	52	24	73	169	23.7	7.0	0.99	1.01	446	62.4	6.12	0.85	1.17
				320♂	39	26	87	150	21.0	5.8	0.81	1.24	473	66.2	5.44	0.76	1.31
Average										6.1	0.85	1.19			5.78	0.81	1.24

Wheat bread.	10	0	10.0	219 ♀	47	10	36	144	14.4	14.4	1.44	0.69	394	39.4	10.9	1.09	0.91
				220 ♂	45	3	24	146	14.6	48.7*	4.87*	0.21*	353	35.3	14.7	1.47	0.68
				221 ♂	48	10	42	159	15.9	15.9	1.59	0.63	412	41.2	9.8	0.98	1.02
				330 ♀	49	9	36	151	15.1	16.8	1.68	0.60	382	38.2	10.6	1.06	0.94
				331 ♂	47	8	46	155	15.5	19.4	1.94	0.52	376	37.6	8.2	0.82	1.22
				332 ♀	46	12	38	155	15.5	12.9	1.29	0.77	392	39.2	10.3	1.03	0.97
Average..										15.9	1.59	**0.64**			10.8	1.08	**0.96**

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* Not counted in average.

TABLE II.

Gain of Body Weight Per Gm. of Ingested Protein Furnished by 25 Per Cent Peanut Bread and Wheat Bread (Approximately a "Straight Flour").

Source of protein.	Protein in food.			Rat.	Initial body weight.	Gain in 4 wks.	Gain in 10 wks.	4 wks. period.					10 wks. period.				
								Total intake.		Intake per gm. of gain.		Gain per gm. of protein.	Total intake.		Intake per gm. of gain.		Gain per gm. of protein.
	From wheat flour.	From peanut flour.	Total protein in diet.					Food.	Protein.	Food.	Protein.		Food.	Protein.	Food.	Protein.	
	per cent	*per cent*	*per cent*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
25 per cent peanut bread.	7.4	9.4	16.8	463 ♀	71	49	75	253	42.6	5.17	0.87	1.15	610	102.5	8.1	1.37	0.73
				464 ♀	68	72	124	267	44.9	3.71	0.62	1.60	707	118.8	5.7	0.96	1.04
				465 ♀	52	58	124	230	38.7	3.97	0.67	1.50	646	108.5	5.2	0.88	1.14
Average										4.28	0.72	**1.42**			6.3	1.07	**0.97**
25 per cent peanut bread.	4.2	5.8	10.0	496 ♀	40	56	94	189	18.9	3.38	0.34	2.96	545	54.5	5.8	0.58	1.73
				497 ♂	36	100	190	273	27.3	2.73	0.27	3.67	817	81.7	4.3	0.43	2.33
				498 ♀	42	57	86	205	20.5	3.60	0.36	2.78	518	51.8	6.0	0.60	1.66
Average										3.24	0.32	**3.14**			5.4	0.54	**1.91**
Wheat bread.	10	0	10	466 ♀	72	24	56	219	21.9	9.13	0.91	1.09	574	57.4	10.2	1.02	0.98
				467 ♂	50	18	50	175	17.5	9.72	0.97	1.03	519	51.9	10.4	1.04	0.96
				468 ♂	62	20	56	202	20.2	10.10	1.01	0.99	538	53.8	9.6	0.96	1.04
Average										9.65	0.96	**1.04**			10.1	1.01	**0.99**

3. Wheat flour (74 per cent extraction) contains sufficient water-soluble vitamine for the normal growth of albino rats.

4. The proteins in the peanut bread were utilized almost twice as well as those contained in the wheat bread.

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A METABOLIC STUDY OF AMYOTONIA CONGENITA.

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PLATES 5 AND 6.

(Received for publication, May 13, 1920.)

The condition of amyotonia congenita is generally defined as a disease beginning at or before birth or in early infancy, probably due to a congenital developmental defect of the lower motor neurons and of the voluntary muscles. Clinically it is characterized by weakness, hypotonia, and quantitatively diminished electrical response in the muscles, usually without disturbance of sensation or mentality. The literature is reviewed by Reuben (1) and Faber (2).

Review of the literature on the subject reveals only three references concerning the chemistry of Oppenheim's disease. Spriggs (3) published the first chemical research, making a study of the creatinine and uric acid excretion. By comparing his results obtained from an experiment with a boy suffering from Oppenheim's disease with a normal boy of the same age and practically the same weight, he showed that the creatinine excretion is diminished to less than one-half the normal figure while the uric acid remains normal. Spriggs statement concerning creatinine was confirmed in 1912 by Gittings and Pemberton (4), who also established a normal calcium retention. The case studied by Powis and Raper (5) which was associated with some nutritional disturbance and acholia was decidedly different from those cases previously mentioned. They found a low creatinine excretion associated with a high creatine excretion, a normal calcium retention, and a relatively high potassium retention.

Our case, W.H., showed the classical symptomatology described by Reuben (1) and Faber (2).

He is 3½ years old and of American nationality. He speaks somewhat slowly with a slight quaver in his voice as though there was difficulty in articulation. At 9 months he was able to sit up if helped and supported but has at no time been able to raise himself voluntarily. When sitting up if his head falls backward he is unable to raise it again. He is inclined to be nervous, and cries easily and for long periods.

The patient appeared to be overnourished, but was flabby with a general lack of muscular tone seeming to involve all the voluntary muscles of the body. His color was good. He was bright and interested in his surroundings. A fine tremor of both hands upon the slightest exertion resulted, but there were no spasms, twitchings, choreaform movements, or convulsions. The patient was able to perform the natural movements with both arms and legs within narrow limits, but only in a weak and uncertain manner.

The hemoglobin was 94 per cent. White blood cells 10,600, red blood cells 4,800,000. Polymorphonuclear cells 41 per cent. Lymph cells 53 per cent. Large mononuclear 1. Transitional 2. Eosinophils 3. There was no response to electrical stimulus under 5 milliamperes. A small piece of muscle was obtained and sections were cut in paraffin. Only a few fibers of normal size were found in the sections. These occurred for the most part in a single group but a few were scattered throughout the section. Nearly all the fibers were greatly reduced in size, the diameter being usually about one-tenth that of the normal. Many muscle fasciculi contained only atrophic fibers. The fibers showed no abnormality except a decrease in size and an indistinctness of the fibrillæ. There was no hyaline degeneration or necrosis. The relative size and the arrangement of the atrophic fibers may be seen in the photomicrographs (Figs. 1, 2, and 3), each of which contains one or more normal fibers.

Treatment.—The patient has had cod liver oil and phosphorus over long periods previous to metabolic study, and has had a thorough test on thyroid and a mixture of thyroid and pituitary glands without any definite improvement. Under careful diet with rather large amounts of protein, with oil rubs, expert massage, and carefully executed regular exercise, and with the support of a light body brace, the patient has shown some slight improvement in muscular status. He suffered an acute attack of pneumonia in March, 1919, but made a normal recovery with no marked change in his general condition.

During the experimental period, which lasted a month, W. H. was kept on a creatinine-free diet. This period which commenced and ended with a fasting period was followed by a dose of charcoal. The method of McCrudden (6) for performing metabolism experiments was rigidly adhered to throughout by a special nurse. The food, feces, and urine were collected for 24 hour periods from 6 a.m. to 6 a.m. The urine was analyzed daily except for January 25th to 26th, 27th to 28th, and February 8th to 9th. On those days the specimens for January 25th to 26th, 27th to 28th, and February 8th to 9th were mixed and the daily average was taken. The feces, which were combined in periods of 3 and 4 days, were analyzed and the average was taken. The food analyses were also made in the same way. On the day the 24 hour period ended, the urine analyses were made, the food and feces made acid with hydrochloric acid, and these were put on the water bath to dry.

Methods of Analysis.

Acetone	Rothera's qualitative, Messinger and Hupper's quantitative.
Acidity	Folin.
Ammonia	Folin macro-aeration.
Ash	Ashing dry solids in muffle.
Chlorides	Volhard-Arnold.
Creatine-creatinine	Folin.
Fat	Soxhlet extraction.
Nitrogen total	Kjeldahl.
Phosphates	Uranium acetate.
Protein	Heller's ring.
Solids total	Drying method.
Sugar	Benedict.
Sulfates	Folin.
Urea	Plimmer and Skelton.
Uric acid	Benedict and Hitchcock.

The volume of the urine (Table I) was reduced to about one-half the normal volume for a child 2 to 3 years of age. This was due to the fact that the child was largely on a vegetable diet and perspired a great deal. To avoid any increase or variation in the nitrogenous elimination by flushing the tissues (7) the fluid intake was kept as high and as nearly constant as possible. As W. H. had no desire for food, it was very difficult to get him to eat or drink. Sometimes 1½ hours were required to feed him a simple meal. Several weeks before this experiment was started he was fasted for 2 days, but even after that he showed no craving for food.

The urine was always acid, and contained no protein or sugar. The indican test was negative, hence the intestinal putrefaction was normal or else no tryptophane or indole was formed.

Acetone was rarely present in the urine and only in small amounts. If this condition is in any way analogous to carbohydrate starvation, an increase of acetone should be expected, at least since there is an apparent breaking down of the tissues. The absence of acetone may be due to the relatively high carbohydrate content of the diet compared with the protein and fat. On examining the urinary sediment the only thing of interest found was the calcium phosphate crystals. These were the dicalcium phosphate crystals which are common in anemia and diseases in which articulations are affected (8). The feces, which varied

TABLE I.

Urine Analyses.

Date.	Volume.	Specific gravity.	Reaction.	Protein.	Sugar.	Indican.	Acetone.	Acidity, expressed in cc. 0.1 N NaOH.	Total N.	Urea N.	NH₃ N.	Uric acid.	Creatine.	Creatinine.	Total creatine and creatinine.	Total solids.	Ash.	NaCl	P₂O₅
1919	*cc.*						*gm.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Jan. 15	255	1.030	Acid.	—	—	—	0.0839	147.68	3.988	2.667	0.227	0.168	0.051	0.263	0.307	17.76	4.812	2.520	0.736
" 16	320	1.020	"	—	—	—	—	108.80	3.506	2.879	0.164	0.168	0.068	0.124	0.183	14.72	5.250	3.490	0.605
" 17	220	1.020	"	—	—	—	—	35.00	2.700	2.128	0.161	0.145	0.046	0.084	0.125	9.98	4.208	2.829	0.373
" 20	290	1.023	"	—	—	—	—	88.64	2.872	2.082	0.159	0.186	0.110	0.084	0.180	13.99	3.856	1.344	0.608
" 21	315	1.020	"	—	—	—	—	61.40	2.822	2.226	0.149	0.159	0.111	0.082	0.178	12.47	5.120	2.479	0.507
" 22	280	1.020	"	—	—	—	—	45.12	2.464	1.854	0.137	0.099	0.074	0.074	0.138	12.45	6.064	3.497	0.483
" 23	290	1.023	"	—	—	—	—	79.20	3.028	2.365	0.136	0.178	0.136	0.091	0.219	14.03	6.550	3.372	0.588
" 24	250	1.023	"	—	—	—	—	108.96	1.803	1.352	0.102	0.145	0.065	0.112	0.168	11.58	5.024	2.644	0.564
" 25	308	1.024	"	—	—	—	—	143.28	4.122	3.368	0.102	0.210	0.143	0.103	0.227	16.20	4.734	2.165	0.843
" 26	280	1.024	"	—	—	—	—	143.28	4.122	3.368	0.102	0.210	0.143	0.103	0.227	16.20	4.734	2.165	0.843
" 27	220	1.025	"	—	—	—	—	68.54	2.028	1.258	0.182	0.122	0.095	0.085	0.167	10.99	4.291	2.074	0.390
" 28	210	1.025	"	—	—	—	—	68.54	2.028	1.258	0.182	0.122	0.095	0.085	0.167	10.99	4.291	2.074	0.390
" 29	220	1.025	"	—	—	—	—	107.52	2.791	2.198	0.202	0.202	0.115	0.088	0.187	12.20	4.048	2.376	0.568
" 31	260	1.025	"	—	—	—	0.0184	100.64	4.250	3.202	0.147	0.206	0.132	0.101	0.215	14.64	6.611	2.928	0.718
Feb. 1	260	1.030	"	—	—	—	—	111.20	3.571	2.958	0.153	0.212	0.102	0.086	0.175	15.97	5.928	3.120	0.737
" 3	240	1.030	"	—	—	—	—	116.00	2.915	2.434	0.229	0.196	0.046	0.080	0.120	14.01	4.780	2.672	0.612
" 4	220	1.020	"	—	—	—	—	62.88	2.502	1.994	0.180	0.152	0.077	0.072	0.138	9.98	4.056	2.424	0.464
" 5	270	1.023	"	—	—	—	—	74.56	2.582	2.159	0.151	0.194	0.090	0.080	0.158	11.95	5.104	2.768	0.551
" 6	140	1.035	"	—	—	—	—	60.48	2.130	1.609	0.115	0.142	0.091	0.085	0.163	8.86	3.204	1.656	0.420
" 7	160	1.035	"	—	—	—	—	79.65	2.412	1.871	0.142	0.144	0.128	0.089	0.200	12.14	4.646	1.764	0.522
" 8	325	1.019	"	—	—	—	—	65.82	2.639	2.208	0.146	0.140	0.104	0.087	0.177	15.27	5.749	2.369	0.572
" 9	230	1.029	"	—	—	—	—	65.82	2.639	2.208	0.146	0.140	0.104	0.087	0.177	15.27	5.749	2.369	0.572
" 10	425	1.016	"	—	—	—	—	84.80	3.344	2.803	0.182	0.162	0.124	0.088	0.196	13.40	6.464	3.740	0.613
" 11	190	1.035	"	—	—	—	+	128.04	3.720	3.032	0.124	0.074	0.130	0.087	0.200	13.68	4.584	1.836	0.714
" 12	210	1.033	"	—	—	—	+	137.20	3.732	3.252	0.217	0.126	0.090	0.091	0.169	15.07	5.068	2.716	0.584
" 13	135	1.033	"	—	—	—	—	59.25	2.119	1.669	0.140	0.151	0.059	0.061	0.113	9.95	3.798	2.142	0.392
" 14	280	1.025	"	—	—	—	—	60.48	3.520	2.302	0.161	0.161	0.087	0.083	0.158	14.64	7.368	3.256	0.680

TABLE II.

Nitrogen, Fat, and Ash Balances.

Date.	Volume.	Feces, dry weight.	Food, dry weight.	Total N intake.	Total N output.	Nitrogen balance.	Total fat intake.	Total fat output.	Fat balance.	Ash intake.	Ash output.	Ash balance.
1919	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Jan. 15	255	24.23	616.50	10.09	11.31	−1.22	71.86	1.79	+70.10	24.05	18.80	+5.25
" 16	320											
" 17	220											
" 20	290	21.55	651.00	10.95	9.21	+0.74	90.71	1.27	+89.44	21.19	17.85	+3.34
" 21	315											
" 22	280											
" 23	290	42.96	851.50	9.79	14.11	−4.32	138.19	4.12	+134.07	23.77	28.67	−4.90
" 24	250											
" 25	308											
" 26	280											
" 27	220	33.87	562.33	9.75	8.59	+1.16	83.70	3.42	+80.28	17.79	18.35	−0.56
" 28	210											
" 29	220											
" 31	260	21.01	355.90	8.17	8.96	−0.80	60.69	1.74	+58.95	15.12	15.32	−0.20
Feb. 1	260											
" 3	240	26.72	545.79	10.96	9.20	+1.76	82.81	2.24	+80.57	16.85	17.55	−0.70
" 4	220											
" 5	270											
" 6	140	33.88	837.90	10.40	11.63	−1.23	105.00	2.35	+102.65	21.63	24.52	−2.89
" 7	160											
" 8	325											
" 9	230											
" 10	425	32.61	426.24	9.70	12.48	−2.78	69.42	2.26	+67.16	18.59	22.26	−3.67
" 11	190											
" 12	210											
" 13	135	70.59	419.30	7.55	10.57	−3.02	48.22	6.63	+41.59	18.44	23.19	−4.74
" 14	280											

in color from light to dark brown, were neutral or slightly acid in reaction and contained hydrobilirubin.

The child weighed 13.49 kilos at the beginning of the experiment and at the end 13.21 kilos. The approximate caloric intake varied from 700 to 1,400 calories per day; the average for the period being 900 or about 53.5 calories per kilo of body weight. According to Sommerfeld (9), this is about 25 calories less per kilo than for a normal child of this age.

Although the nitrogen balance (Table II) for the entire period was negative, there were several periods during the month in which nitrogen equilibrium was maintained. For the month the loss of nitrogen was 9.7 gm.

Camerer (10) has established 0.695 gm. of P_2O_5 as normal urinary elimination for boys of 3 years of age. According to this the average daily phosphorus metabolism is normal.

The ash balance (Table II) for the period is negative.

Since protein is the source of the urinary sulfur as well as nitrogen (Tables III and IV), a parallelism, although not exact, can be expected in the daily variations of nitrogen and sulfur. The figures for neutral sulfur and undetermined nitrogen fulfill this expectation. The inorganic sulfates are slightly subnormal, while the organic and especially the neutral sulfur percentages are high, as compared with Schwarz's figures (11) for a normal 5 year boy (86 to 87 per cent inorganic sulfur, 4.0 per cent organic sulfur, and 7.8 per cent neutral sulfur). Weiss (12) thinks that most of the neutral sulfur is endogenous, also that any condition which causes a rise in the neutral sulfur can be attributed to destruction of body protein.

In the blood analysis Lyman's method (13) was used for the determination of calcium; the other methods are those described by Gradwohl (14), except that a Duboscq colorimeter replaced the Hellige. The low urea figure which occurs in a fasting condition is the most significant (Table V). After 17 hours fast in adults Schwartz and McGill (15) found 5 to 7.1 mg. of urea N per 100 cc. of blood. Fasting also increases the blood sugar. No figures seem to be available for children of this age. Since the blood analysis applies to only one specimen of blood (sugar excepted) we do not lay stress on an interpretation of the results.

TABLE III.

Urinary Nitrogen Partition.

Date.	Total N.	Urea N.	NH₃ N.	Creatine N.	Creatinine N.	Uric acid N.	Rest N.	Per cent of total nitrogen.					
								Urea N.	NH₃ N.	Creatine N.	Creatinine N.	Uric acid N.	Rest N.
1919	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Jan. 15	3.988	2.667	0.227	0.014	0.097	0.056	0.925	66.88	5.70	0.36	2.44	1.40	23.19
" 16	3.506	2.879	0.164	0.019	0.046	0.056	0.341	82.11	4.69	0.55	1.31	1.60	9.73
" 17	2.700	2.128	0.161	0.013	0.031	0.048	0.317	78.82	5.97	0.39	1.17	1.80	11.77
" 20	2.872	2.082	0.159	0.031	0.031	0.062	0.504	72.51	5.55	1.08	1.09	2.16	17.58
" 21	2.822	2.226	0.149	0.031	0.030	0.053	0.331	78.87	5.31	1.11	1.08	1.87	11.75
" 22	2.464	1.854	0.137	0.020	0.027	0.032	0.390	75.29	5.59	0.84	1.12	1.33	15.82
" 23	3.028	2.365	0.136	0.038	0.033	0.059	0.394	78.11	4.50	1.27	1.11	1.96	13.03
" 24	1.803	1.352	0.102	0.018	0.041	0.048	0.239	74.97	5.68	1.02	2.31	2.68	13.30
" 25	4.122	3.368	0.102	0.040	0.038	0.070	0.502	81.71	2.50	0.98	0.93	1.69	12.19
" 26	4.122	3.368	0.102	0.040	0.038	0.070	0.502	81.71	2.50	0.98	0.93	1.69	12.19
" 27	2.028	1.258	0.182	0.026	0.031	0.040	0.488	62.20	9.00	1.33	1.55	2.00	24.08
" 28	2.028	1.258	0.182	0.026	0.031	0.040	0.488	62.20	9.00	1.33	1.55	2.00	24.08
" 29	2.791	2.198	0.202	0.032	0.032	0.067	0.257	78.76	7.26	1.16	1.17	2.41	9.23
" 31	4.250	3.202	0.147	0.037	0.037	0.068	0.757	75.34	3.47	0.87	0.88	1.61	17.81
Feb. 1	3.571	2.958	0.153	0.028	0.032	0.070	0.327	82.84	4.29	0.81	0.90	1.98	9.17
" 3	2.915	2.434	0.229	0.013	0.029	0.065	0.143	83.51	7.87	0.45	1.02	2.24	4.91
" 4	2.502	1.994	0.180	0.021	0.026	0.050	0.227	79.70	7.23	0.86	1.08	2.03	9.10
" 5	2.582	2.159	0.151	0.025	0.029	0.064	0.151	83.62	5.87	0.99	1.16	2.50	5.85
" 6	2.130	1.609	0.115	0.025	0.031	0.047	0.300	75.55	5.44	1.21	1.48	2.23	14.08
" 7	2.412	1.871	0.142	0.036	0.033	0.048	0.281	77.58	5.91	1.49	1.37	1.98	11.64
" 8	2.639	2.208	0.146	0.029	0.032	0.046	0.176	83.67	5.56	1.11	1.23	1.77	6.67
" 9	2.639	2.208	0.146	0.029	0.032	0.046	0.176	83.67	5.56	1.11	1.23	1.77	6.67
" 10	3.344	2.803	0.182	0.035	0.032	0.054	0.236	83.82	5.45	1.04	0.98	1.61	7.07
" 11	3.720	3.032	0.124	0.036	0.032	0.024	0.468	81.52	3.35	0.99	0.87	0.67	12.60
" 12	3.732	3.252	0.217	0.025	0.033	0.042	0.161	87.12	5.83	0.68	0.91	1.13	4.33
" 13	2.119	1.669	0.140	0.016	0.022	0.050	0.220	78.75	6.60	0.79	1.08	2.38	10.39
" 14	3.520	2.302	0.161	0.024	0.030	0.053	0.947	65.40	4.59	0.70	0.88	1.51	26.92
Daily average.....	2.976	2.323	0.157	0.027	0.035	0.053	0.379	77.64	5.57	0.94	1.21	1.85	12.78

TABLE IV.

Urinary Sulfur Partition.

Date.	Total SO₃.	Inorganic SO₃.	Organic SO₃.	Neutral SO₃.	Per cent of total SO₃.		
					Inorganic SO₃.	Organic SO₃.	Neutral SO₃.
1919	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Jan. 15	1.385	0.769	0.149	0.467	55.52	10.75	33.72
" 16	0.702	0.602	0.018	0.081	85.75	2.56	11.54
" 17	0.495	0.427	0.002	0.066	86.26	0.41	13.22
" 20	0.589	0.435	0.035	0.118	73.85	5.94	20.03
" 22	0.521	0.394	0.020	0.106	75.62	3.84	20.34
" 23	0.687	0.565	0.034	0.087	82.24	4.94	12.66
" 24	0.695	0.597	0.029	0.068	85.90	4.17	9.78
" 25	0.846	0.738	0.037	0.070	87.23	4.37	8.27
" 26	0.846	0.738	0.037	0.070	87.23	4.37	8.27
" 27	0.585	0.506	0.033	0.044	86.49	5.64	7.52
" 28	0.585	0.506	0.033	0.044	86.49	5.64	7.52
" 31	0.781	0.652	0.041	0.087	83.49	5.25	11.14
Feb. 1	0.712	0.575	0.079	0.057	80.76	11.09	8.00
" 3	0.615	0.486	0.037	0.092	79.02	6.06	14.96
" 4	0.509	0.431	0.020	0.058	84.67	3.92	11.39
" 5	0.548	0.432	0.018	0.097	78.83	3.28	17.70
" 6	0.355	0.290	0.031	0.032	81.69	8.73	9.01
" 8	0.582	0.502	0.047	0.032	86.25	8.07	5.49
" 9	0.582	0.502	0.047	0.032	86.25	8.07	5.49
" 11	0.660	0.575	0.041	0.043	87.12	6.21	6.51
" 12	0.635	0.544	0.043	0.047	85.67	6.77	7.40
" 13	0.343	0.278	0.030	0.034	81.05	8.75	9.91
" 14	0.639	0.553	0.038	0.048	86.54	5.96	7.51
Daily average					82.34	5.86	11.63

TABLE V.

Blood Analysis.

	per cent
6–21 Sugar	0.13
7–1 "	0.14
11–27 "	0.15

	mg. per 100 cc. of blood.
Creatinine	1.50
Creatine	5.45
Calcium	7.10
Urea N	3.50

SUMMARY.

The significant facts observed in the metabolism of a case of amyotonia congenita were:

1. A lowered creatinine excretion, in addition to the excretion of creatine on a low protein diet.
2. A normal uric acid excretion; therefore no nucleoprotein broken down.
3. An increased rest nitrogen, accompanied by an increased neutral sulfur.
4. A normal phosphorus excretion; therefore no bone disintegration.
5. A lowered chloride excretion.

We wish to thank Dr. J. P. Sedgwick, who suggested the problem, for his kindly interest in the research.

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EXPLANATION OF PLATES.

PLATE 5.

FIG. 1. Group of normal fibers with a number of atrophic fibers in the same fasciculus. Fasciculus of atrophic fibers on right.

PLATE 6.

FIG. 2. High power photomicrograph of Fig. 1, showing atrophic and normal fibers in the same fasciculus.

FIG. 3. Fasciculus of atrophic fibers under moderately high magnification. One normal fiber.

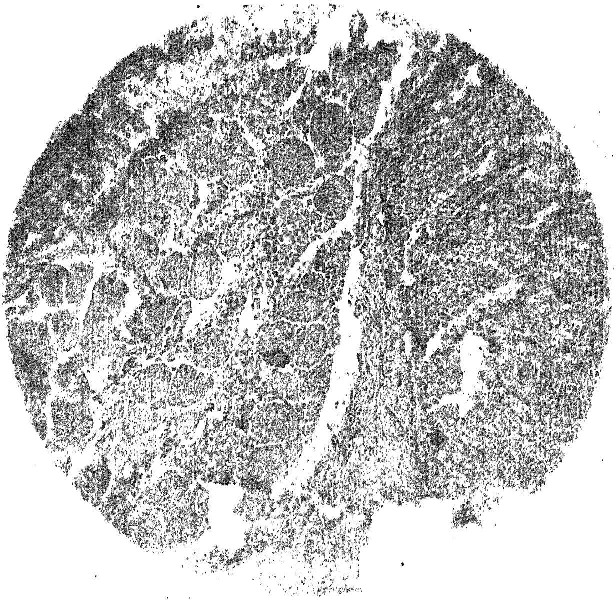

FIG. 1.

(Ziegler and Pearce: Metabolism of amyotonia congenita.)

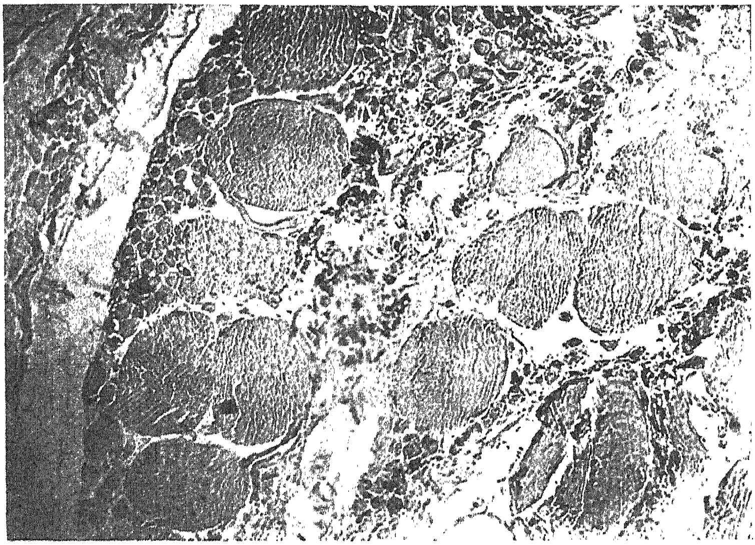

FIG. 2.

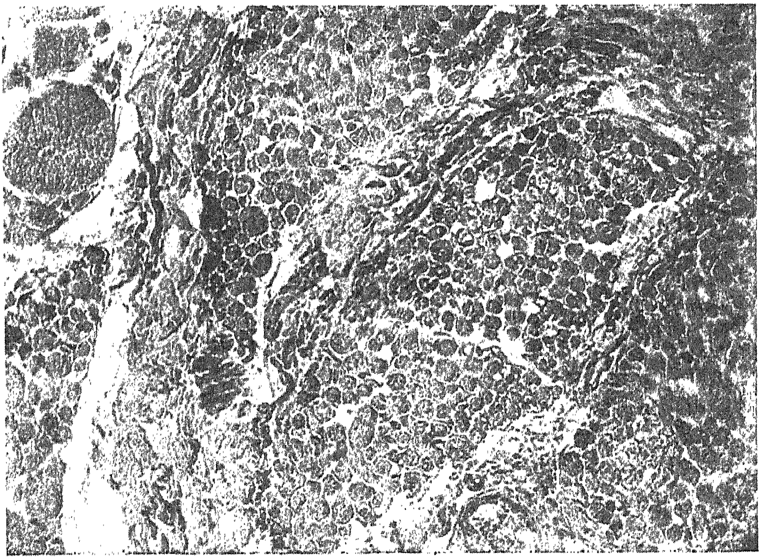

FIG. 3.

(Ziegler and Pearce: Metabolism of amyotonia congenita.)

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